Selective EGF-Receptor Inhibition in CD4$^+$ T Cells Induces Anergy and Limits Atherosclerosis

Lynda Zeboudj, MS[a*]; Mikael Maître, MS[a*]; Lea Guyonnet, PhD[a]; Ludivine Laurans, MS[a]; Jeremie Joffre, MD, PhD[a]; Jeremie Lemarie, MD, PhD[a]; Simon Bourcier, MD[a]; Wared Nour-Eldine, MS[a]; Coralie Guérin, PhD[b]; Jonas Friard, MS[c]; Abdelilah Wakkach, PhD[c]; Elizabeth Fabre, MD[d]; Alain Tedgui, PhD[a]; Ziad Mallat, MD, PhD[a,e]; Pierre-Louis Tharaux, MD, PhD[a], Hafid Ait-Oufella, MD, PhD[a,f]

[a]Inserm U970, Paris Cardiovascular Research Center, Paris, France, Université René Descartes Paris; [b]Luxembourg Institute of Health Department of Infection and Immunity, Luxembourg ; [c]CNRS, LP2M, UMR 7370, Faculté de médecine, Université de Nice Sophia Antipolis, Nice France; [d]Department of Medical Oncology, Hopital Europeen G. Pompidou, AP-HP, Paris, France; [e]Department of Medicine, Division of Cardiovascular Medicine, University of Cambridge, Cambridge, United Kingdom; [f]Service de Réanimation Médicale, Hôpital Saint-Antoine, AP-HP, Université Pierre-et-Marie Curie, Paris
[*]The authors contributes equally to the work

Short title: EGF-R blockade in T cells reduces atherosclerosis
Competing Financial interests: None
Disclosures: None

Sources of funding: This work was supported by Institut National de Santé et de la Recherche Médicale (INSERM), research grants ANR-08-EBIO-003 (PL.T) from l'Agence Nationale de la Recherche (ANR), British Heart Foundation (Z.M).

***Correspondence**
Hafid Ait-Oufella, MD, PhD
Inserm U970
Paris Cardiovascular Research Center
56, rue Leblanc Paris, France
Université René Descartes Paris 5
Telephone: +33612011940
Fax: +3314463186
E-mail: hafid.aitoufella@inserm.fr

ABSTRACT
Background: Several epidermal growth factor receptor (EGFR) inhibitors have been successfully developed for the treatment of cancer, limiting tumor growth and metastasis. EGFR is also expressed by leukocytes, but little is known about its role in the modulation of the immune response.
Objectives: We aimed to determine whether EGFR expressed on $CD4^+$ T cells is functional, and to address the consequences of EGFR inhibition in atherosclerosis, a T cell-mediated vascular chronic inflammatory disease.
Methods and. Mouse $CD4^+$ T cells expressed EGFR, and the EGFR tyrosine kinase inhibitor AG-1478 blocked in vitro T cell proliferation and Th1/Th2 cytokine production.
Results: In vivo, treatment of *Ldlr*$^{-/-}$ mice with the EGFR inhibitor Erlotinib induced T cell anergy, reduced T cell infiltration within atherosclerotic lesions and protected against atherosclerosis development and progression. Selective deletion of EGFR in $CD4^+$ T cells resulted in decreased T cell proliferation and activation both in vitro and in vivo, as well as reduced IFN-γ, IL-4, IL-2 production. Atherosclerotic lesion size was reduced by 2-fold in irradiated *Ldlr*$^{-/-}$ mice reconstituted with bone marrow from *Cd4-Cre/Egfr*$^{lox/lox}$ mouse, compared to *Cd4-Cre/Egfr*$^{+/+}$ chimeric mice, after 4, 6 and 12 weeks of high fat diet, associated with marked reduction in T cell infiltration in atherosclerotic plaques. Human blood T cells expressed EGFR and EGFR inhibition reduced T cell proliferation both in vitro and in vivo.
Conclusions. EGFR blockade induced T cell anergy in vitro and in vivo, and reduced atherosclerosis development. Targeting EGFR may be a novel strategy to combat atherosclerosis.

Key words: Atherosclerosis, immunity, inflammation, lymphocyte.

Condensed Abstract: $CD4^+$ T cell adaptive immunity, in response to lipoprotein accumulation in the arterial wall, is involved in the development of atherosclerosis. Using several complementary approaches, we identified for the first time a critical role of EGFR in $CD4^+$ T cell homeostasis, in both mice and humans. EGFR genetic invalidation or pharmacological blockade impaired T cell activation, proliferation, cytokine production and reduced both atherosclerosis development and progression. Our findings indicate that EGFR inhibitors, widely used in patients with cancer, are unlikely to worsen the risk of cardiovascular disease, and even more suggest that EGFR may constitute a novel therapeutic target in atherosclerosis-related diseases.

Abbreviations
CD, Cluster of Differentiation
EGF, Epidermal Growth factor
IL-, Interleukin
LDL, Low Density Lipoprotein
Th, T Helper
TKI, tyrosine kinase inhibitors

Introduction

Epidermal Growth Factor Receptor (EGFR) is a cell membrane-bound receptor with tyrosine kinase activity involved in the control of major signaling pathways, including cell survival, proliferation and migration. EGFR overexpression, autocrine ligand stimulation, or constitutively active receptor mutants (1,2) can lead to dysregulation of this fine-tuned signaling system, resulting in a variety of pathophysiological disorders and promoting cancer development. Six EGFR ligands have been described, including Epidermal Growth Factor (EGF), Heparin Binding-Epidermal Growth Factor (HB-EGF), Amphiregulin and Transforming Growth Factor-α. Extracellular ligand binding causes dimerisation of EGFR, which becomes autophosphorylated at distinct tyrosine residues. In addition, EGFR could be transactivated in the absence of a specific ligand through G-protein-coupled receptor activation (3).

EGFR has been extensively explored in cancer. Human and experimental studies showed that EGFR activation on tumor cells ultimately leads to cell proliferation, invasion, and migration, as well as promoting angiogenesis and inhibiting apoptosis (4). Targeting of EGFR by either neutralizing monoclonal antibodies or small-molecule tyrosine kinase inhibitors (TKI) have been shown during the last ten years to be a successful therapeutic strategy in cancer setting (5,6). However, EGFR expression and functions have been poorly investigated in non-tumoral cells. Some authors described an expression in circulating leucocytes (7,8) but little is known about its role in the modulation of the immune response.

Atherosclerosis is an inflammatory disease driven by innate and adaptive immunity, in which $CD4^+$ T cells play a pathogenic role. Interestingly, EGFR ligands, including HB-EGF have been detected in human atherosclerotic plaques (9). The aim of this study was to ascertain the expression of EGFR in human and mouse $CD4^+$ T cells, and to investigate the effects of

EGFR blockade on $CD4^+$ T cell functions using pharmacological inhibitors and cell-specific genetic deletion in mouse models of atherosclerosis.

Methods

Animals

Experiments were conducted according to the guidelines formulated by the European Community for experimental animal use (L358-86/609EEC), and were approved by the Ethical Committee of INSERM and the French Ministry of Agriculture (agrement no. A75-15-32). To generate a cell-specific knockout of *Egfr* in $CD4^+$ T cells, we crossbred mice carrying a *Cd4Cre* allele with mice carrying a floxed *Egfr* allele. All animals have been backcrossed more than ten generations on *C57bl/6* background. Ten-week old male C57BL/6 $Ldlr^{-/-}$ mice were put on a high fat diet for 8 weeks and were treated with the oral (daily gavage), specific EGFR tyrosine kinase inhibitor, erlotinib (15mg/kg/day). For bone marrow transplantation experiments, 10-week old male *C57bl/6* $Ldlr^{-/-}$ mice were subjected to medullar aplasia by lethal total body irradiation (9.5 grays). The mice were repopulated with an intravenous injection of bone marrow cells isolated from femurs and tibias of sex-matched C57BL/6 *Cd4Cre* $Egfr^{+/+}$ mice or *Cd4Cre* $Egfr^{lox/lox}$ littermates. After 4 weeks of recovery, mice were fed a pro-atherogenic diet containing 15% fat, 1.25% cholesterol and 0% cholate for 4, 6, and 12 weeks.

Extent and composition of atherosclerotic lesions

Plasma cholesterol was measured using a commercial cholesterol kit (Biomérieux). The heart of mice was removed and successive 10-µm transversal sections of aortic sinus were obtained. Lipids were detected using Red Oil staining. The presence of T cells was studied using specific antibodies, as previously described (polyclonal anti-CD3, DAKO) (10). *Egfr* was detected in cells and lesions using a rabbit polyclonal anti-Phospho-Egfr (Cell Signaling, Boston,

USA). For human staining, an anti-EGFR antibody (clone 31G7, AbCys S.A.) was used. At least 4 sections per mouse were examined for each immunostaining, and appropriate negative controls were used. Morphometric studies were performed using Histolab software (Microvisions) (10).

Spleen cell recovery and purification

Spleen cells were purified according to standard protocols as follows. $CD4^+$ T cells were negatively selected using a cocktail of antibody-coated magnetic beads from Miltenyi Biotech (anti-CD8a, anti-CD11b, anti-CD45R, anti-DX5, anti-ter 119), according to manufacturer's instructions, yielding $CD4^+$ cells with >95% purity. $CD11c^+$ cells were positively selected with biotin-conjugated anti-CD11c mAb (7D4, PharMingen), streptavidin microbeads (Miltenyi Biotec), followed by 2 consecutive magnetic cell separations using LS columns (Miltenyi Biotec), yielding $CD11c^+$ cells with >80% purity.

$CD4^+$ T cell culture and cytokine assays

Cells were cultured in RPMI 1640 supplemented with Glutamax, 10% FCS, 0.02 mM β-mercaptoethanol and antibiotics. For cytokine measurements, $CD4^+$ T cells were cultured at $1x10^5$ cells/well for 48 hours in anti-CD3-coated microplates (10 μg/ml) or with Concanavalin A (Sigma, 10 μg/ml). In some experiments, $CD4^+$ T cells were stimulated with purified soluble CD3-specific antibody (1 μg/ml, Pharmingen) in the presence of antigen-presenting cells purified on CD11c-coated magnetic beads (Miltenyi Biotech). IL-2, IL-4, IL-10 and IFN-γ productions in the supernatants were measured using specific ELISAs (R&D Systems).

Other methods (Cell culture, proliferation assays, cytosolic calcium recording, flow cytometry and western Blot) are available in the Online Appendix.

Statistical analysis

Values are expressed as median and percentiles (25^{th}-75^{th}) in the text. Differences between values were evaluated using non-parametric Mann-Whitney or Kruskal-Wallis test. Values of $p<0.05$ were considered significant. All these analysis were performed using GraphPad Prism version 5.0b for Mac (GraphPad Software). No adjustments were made for multiple pair-wise comparisons.

Based on preliminary experiments, we assumed that EGFR blockade induces a 40% reduction of atherosclerosis. With a standard deviation of plaque size estimated at 30%, the inclusion of 8 mice/group was sufficient to detect a significant difference between groups with 80% power.

Results

EGFR in mouse $CD4^+$ T cells: expression and signaling pathways

Using immunocytostaining, we detected EGFR expression by splenocytes and found that EGFR co-localized, non-exclusively, with CD4+ T cells (**Figure 1A**). In addition, EGFR was present in mouse atherosclerotic lesions and co-localized with $CD4^+$ T cells (**Figure 1B**). *In vitro*, anti-CD3- and concanavalin A-induced activation of purified $CD4^+$ T cells caused EGFR phosphorylation after 60 minutes of stimulation (**Figure 1C**). AG-1478, a pharmacological inhibitor of tyrosine kinase, blocked EGFR phosphorylation in stimulated T cells (**Figure 1C**) and significantly reduced ERK1/2 phosphorylation (**Figure 1D**, Online Figure 1), but had no effect on AKT (Online Figure 1). In addition, we found that EGFR inhibition blocked cytoplasmic calcium increase following anti-CD3/CD28 stimulation (**Figure 1E**). In summary, anti-CD3 stimulation of $CD4^+$ T cells leads to EGFR transactivation that was efficiently inhibited by AG-1478.

Effects of pharmacological inhibition of EGFR on atherosclerosis

To address the role of EGFR on T cell functions, splenic $CD4^+$ T cells were purified and stimulated *in vitro*. EGFR activity was inhibited using AG-1478 at different concentrations. EGFR inhibition significantly reduced T cell proliferation following non-antigen-specific (**Figure 2A**) and antigen-specific (**Figure 2B**) stimulation in a dose-dependent manner without any effect on T cell apoptosis (**Figure 2C**) or nucleus-cytoplasm organization (Online Figure 2). Pharmacological inhibition of EGFR significantly reduced intracellular IFN-γ production by $CD4^+$ T cells in response to anti-CD3 (**Figure 2D**) and concanavalin stimulation (**Figure 2E**). This was confirmed by ELISA in the supernatants of cultured splenic $CD4^+$ T cells, showing that EGFR inhibition significantly reduced the production of IFN-γ (**Figure 2 F, G**), reduced the production of IL-2 (supplemental figure 3), as well as that of IL-4 (Figure 2H), in a dose-dependent manner, but had no effect on IL-10 production that was very low (**Figure 2I**).

To investigate the *in vivo* consequences of EGFR pharmacological inhibition, $Ldlr^{-/-}$ male mice were put on a high fat diet during 8 weeks and were orally treated with erlotinib (15mg/kg/day) (**Figure 3A**). At sacrifice, animal weight (Online Figure 4A) and plasma cholesterol level (**Figure 3B**) were not different between groups, but there was a significant reduction of spleen size (supplemental figure 4B) and splenocyte number (**Figure 3C**) in the erlotinib-treated group. Splenic $CD4^+$ T cells from erlotinib-treated group were characterized by a significant decrease in CD25 (Online Figure 5A) and $CD44^{high}$ expression (Supplemental figure 5B), suggesting reduced *in vivo* activation. Erlotinib had no effect on $CD4^+CD25^{high}Foxp3^+$ regulatory T cell population (Online Figure 5C). Splenic purified $CD4^+$ T cells from erlotinib-treated group were characterized by a reduction of proliferation *ex vivo* (**Figure 3D**) and a reduction of Th1 (IFN-γ) and Th2 (IL-4) cytokine production with no effect on IL-10 (**Figure 3E**). Immunohistochemistry analysis revealed that erlotinib treatment induced

a 70% reduction of T cell infiltration within atherosclerotic lesions (P=0.01) (Figure 3F), which was associated with 37% reduction of atherosclerotic lesion size in the aortic sinus (167 [91-190] $x10^3$ in treated mice versus 267 [180-333] $x10^3$ μm^2 in control mice, P<0.05) (**Figure 3G**).

In the vast majority of cases, patients in need of an anti-atherosclerotic therapy already have established atherosclerotic plaques. Thus, we examined the effects of EGFR blockade on the progression of established atherosclerotic plaques in mice. Six-week old *Ldlr*$^{-/-}$ female mice were put on a high fat diet during 8 weeks and then were orally treated with a placebo or erlotinib (15mg/kg/day) during 8 weeks (**Figure 3H**). At sacrifice, animal weight (data not shown) and plasma cholesterol levels (Online Figure 6C) were not different between groups. In Erlotinib-treated group, we observed a marked reduction in atherosclerotic lesion size both in the aortic sinus (176 [159-218] $x10^3$ versus 269 [193-343] $x10^3$ μm^2, P<0.05) (Online Figure 6A & 6B) and along the thoracic aorta (14.0 [12.4-14.9] versus 20.7 [15.2-22.3] %, P<0.05) (**Figure 3I**). More importantly, atherosclerosis plaque size increased between baseline 14-week old mice and 22-week old mice in the placebo group, but did not progress in the group receiving Erlotinib (**Figure 3I** and Online Figure 6), suggesting that Erlotinib treatment blocked atherosclerosis progression.

EGFR in human CD4$^+$ T cells: expression and functions

To evaluate the clinical relevance of our findings, we investigated the expression of EGFR in human blood T cells. We performed immunocytostaining of purified blood CD4+ T cells and found that EGFR was expressed and clustered in the membrane of CD4$^+$ T cells after Concanavalin-induced activation (**Figure 4A**). To investigate the functions of human EGFR, we purified circulating CD4+ T cells from healthy donors and performed *in vitro* proliferation tests. CD3/CD28-coated beads stimulation induced T cell proliferation and EGFR inhibition using

AG-1478 or Cetuximab, an EGFR neutralizing mAb, significantly decreased T cell proliferation (**Figure 4B & 4C**). This result was confirmed *in vivo* in patients with lung cancer. Circulating T cells were isolated from 3 patients before and one month after oral erlotinib treatment. Interestingly, T cell proliferation was lower after erlotinib treatment (**Figure 4D**).

Cell-specific genetic invalidation of Egfr in CD4$^+$ T cells

Erlotinib administration induced T cell anergy and reduced atherosclerosis development. As the expression of EGFR is ubiquitous, we next assessed the specific role of EGFR activation in CD4$^+$ T cells in erlotinib-induced atheroprotection. We bred mice carrying a *Cd4-Cre* allele with mice carrying a floxed *Egfr* allele and generated *Cd4-Cre/Egfr$^{lox/lox}$* mice. The deletion of EGFR specifically in CD4$^+$ T cells was confirmed by immunocytostaining (Online Figure 7). We purified splenic CD4$^+$ T cells from control *Cd4-Cre/Egfr$^{+/+}$* and *Cd4-Cre/Egfr$^{lox/lox}$* mice and performed functional tests. *In vitro*, in agreement with experiments using AG-1478, proliferation of CD4$^+$ T cells from *Cd4Cre Egfr$^{lox/lox}$* mice was significantly decreased compared with wild-type cells (**Figure 5A**), and their production of Th1 (**Figure 5B**) and Th2 (**Figure 5C**) cytokines was reduced. There was no difference in IL-10 production (**Figure 5D**). Similar reduction in cell proliferation and cytokine production was observed when EGFR-deficient CD4$^+$ T cells were co-incubated with CD11c$^+$ antigen presenting cells (Figure 5A-D). Intracellular staining by flow cytometry confirmed the specific reduction of IFN-γ production by CD4$^+$ (**Figure 5E**) but no by CD8$^+$ T cells. Apoptosis susceptibility was significantly lower in EGFR-deficient CD4$^+$ T cells (Online Figure 8). To address the *in vivo* role of EGFR in CD4$^+$ T cell proliferation, we transferred into *Apoe$^{-/-}$/Rag2$^{-/-}$* mice 20.10^6 CD4$^+$ T cell–depleted splenocytes resupplemented with 8.10^6 purified *CFSE*-labeled CD4$^+$ T cells from either *Cd4-Cre/Egfr$^{+/+}$* or *Cd4-Cre/Egfr$^{lox/lox}$* mice. The proliferation of adoptively transferred cells was visualized by flow cytometric analysis

of *CFSE*-labeled $CD4^+$ T cells. At day 10 after transfer, we found that $CD4^+$ T specific deletion of EGFR limited T cell proliferation in the spleen and lymph nodes (**Figure 5F**).

Impact of $CD4^+$ T cell specific invalidation of Egfr on atherosclerosis

To address the consequences of these findings in the context of atherosclerosis, we performed bone marrow transplantation experiments using either *Cd4-Cre/Egfr*$^{+/+}$ or *Cd4-Cre/Egfr*$^{lox/lox}$ littermate bone marrow to repopulate lethally irradiated *Ldlr*$^{-/-}$ mice. After 4 weeks of recovery and additional 4 weeks on high fat diet, animals were sacrified. We did not observe any difference in animal or spleen weights, but a 32% reduction in the number of splenocytes in chimeric *Cd4-Cre/Egfr*$^{lox/lox}$ group (P=0.05). Leukocyte populations (neutrophils, monocytes, B cells, $CD8^+$ T cells) were not different between groups, either in the blood or in the spleen (data not shown). Splenic $CD4^+$ T cell subset was not different between chimeric *Cd4-Cre/Egfr*$^{+/+}$*/Ldlr*$^{-/-}$ and *Cd4-Cre/Egfr*$^{lox/lox}$*/Ldlr*$^{-/-}$ mice (Figure 6A) but the $CD4^+CD25^{high}FoxP3^+$ regulatory T cell (Tregs) population was slightly reduced in the group with CD4 specific deletion of EGFR (-14%, P<0.05; **Figure 6B**). Splenic $CD4^+$ T cells from *Cd4-Cre/Egfr*$^{lox/lox}$*/Ldlr*$^{-/-}$ were characterized by a significant decrease in CD69 (**Figure 6C**) and $CD44^{high}$ expression (**Figure 6D**), suggesting reduced *in vivo* activation. Functional tests were performed *ex vivo* in purified splenic $CD4^+$ T cells. The suppressive function of Tregs was preserved in *Cd4-Cre/Egfr*$^{lox/lox}$*/Ldlr*$^{-/-}$ mice (**Figure 6E**) but the proliferation of $CD4^+$ T cells in response to CD3 stimulation was reduced compared to cells from control group (figure 6F). Finally, we also observed a > 2-fold decrease in IFN-γ and IL-4 production in the supernatant of anti-CD3-stimulated $CD4^+$ T cells from *Cd4-Cre/Egfr*$^{lox/lox}$*/Ldlr*$^{-/-}$ mice compared with *Cd4-Cre/Egfr*$^{+/+}$*/Ldlr*$^{-/-}$ mice (**Figure 6G**).

As shown in **Figure 7A**, after 4 weeks on high fat diet, *Egfr* invalidation in $CD4^+$ T cells led to a 36% decrease in atherosclerotic lesion size in the aortic sinus compared with controls (25 [16-30] $x10^3$ μm^2 vs 34 [31-57] $x10^3$ μm^2 in *Cd4-Cre/Egfr*$^{lox/lox}$/*Ldlr*$^{-/-}$ and *Cd4-Cre Egfr*$^{+/+}$/*Ldlr*$^{-/-}$, respectively, P=0.03). The reduction of atherosclerosis was confirmed after 6 (-39%, P=0.02) and 12 weeks (-43%, P=0.02) of high fat diet (**Figure 7A**). There were no significant differences in plasma cholesterol levels between the groups (Online Figure 9).

We finally analyzed T cell infiltration within the lesions. We found a decrease in T cell number in lesions of *Cd4Cre Egfr*$^{lox/lox}$ → *Ldlr*$^{-/-}$ compared to *Cd4Cre Egfr*$^{+/+}$ → *Ldlr*$^{-/-}$ mice (**Figure 6B**) but no difference in macrophage infiltration (**Online Figure 10).**

Discussion

Using several complementary approaches, we identified a critical role of EGFR in $CD4^+$ T cell homeostasis, in both mice and humans. EGFR genetic invalidation or pharmacological blockade impaired T cell activation, proliferation, cytokine production and reduced atherosclerosis development (**Central Illustration**).

Using immunocytostaining and immunohistochemistry, we found that splenic $CD4^+$ T cells and blood human T cells express EGFR, especially in response to CD3 or concanavalin A stimulation. Immunofluorescent staining showed, after anti-CD3 stimulation, that EGFR clustered on the cell membrane and was phosphorylated. In addition, tissue T cells within atherosclerotic plaques express EGFR. Our results are in line with those of Zaiss et al. who detected both *Egfr* mRNA expression and EGFR protein in purified $CD4^+$ T cells (8).

Our findings indicate that EGFR signaling is crucial in $CD4^+$ T cell homeostasis in both human and mouse. Pharmacological inhibition using TKIs (AG-1478 or erlotinib), neutralizing antibodies or $CD4^+$ T cell specific genetic invalidation of *Egfr* markedly reduced *in vitro* and *in*

vivo cell proliferation and Th1/Th2/Th17 cytokine production. A similar observation has been reported in a mouse model of graft versus host disease with a reduction of Th1 and Th2 cytokine production in erlotinib-treated animals (11). In addition, we observed that EGFR inhibition/invalidation reduced T cell infiltration within atherosclerotic lesions suggesting a modulation of T cell migration. This might be accounted for reduced chemokine production as described in a model of skin inflammation, in which TKI treatment decreased Ccl-17, Ccl-21 and Ccl-27 production (11). Reduction of T cell infiltration in the vascular wall of *Cd4Cre Egf-r* $^{lox/lox}$ animals might also be due to altered cell motility resulting from impairment of cytoskeleton reorganization (12). A large body of evidence in cancer highlighted the role of EGFR signaling in epithelial-mesenchymal transition and invasion/migration of normal and malignant epithelial cells (13). Recently, Tai et al. showed that EGFR/Src-signaling triggers the tyrosine phosphorylation of β4 integrin, which, in turn, activated FAK, a kinase involved in cytoskeleton reorganization (14). In our study, we did not observe any side effect due to EGFR blockade, including survival of Erlotinib-treated or *Cd4 Cre Egfr*$^{lox/lox}$ *mice*. In addition, we did not find any difference in weight or infection susceptibility between groups. These observations suggest that EGFR inhibition modulates the immune response but does cause full immunosuppression.

EGFR inhibition/invalidation did not affect cell death susceptibility but induced a global $CD4^+$ T cell anergy. The mechanisms of anergy induced by EGFR inhibition likely involved MAP kinase signaling pathway as suggested by reduced Erk phosphorylation in $CD4^+$ T cells treated with AG 1478. This is an agreement with studies by Luo et al. showing that erlotinib caused G0/G1 arrest and suppressed the phosphorylation of c-Raf, Erk in activated T cells (15). We also showed that EGFR pharmacological blockade negatively impacted on intracellular calcium signaling in T cells confirming previous reports on cancer context. Bryant et al. showed

on glioma tumor cell lines that tyrosine kinase inhibitors including erlotinib and gefinitib limited the acute cytoplasmic release of calcium from the endoplasmic reticulum in response to EGF (16).

A recent study suggested that EGFR and one of its ligands, amphiregulin, play a specific role in Treg suppressive functions. In mouse models of atherosclerosis, Treg deficiency, obtained by *Foxp3, Cd28* or *Cd80/86* genetic invalidation, increased T cell activation and accelerated vascular disease (17-19). However, in our study, the genetic invalidation of *Egfr* in $Ldlr^{-/-}$ chimeric mouse model had no effect on the suppressive function of Tregs. We only observed a slight reduction in Treg pool in chimeric $Cd4\text{-}Cre/Egfr^{lox/lox}/Ldlr^{-/-}$ compared with $Cd4\text{-}Cre/Egfr^{+/+}/Ldlr^{-/-}$ mice. Our results show that EGFR inhibition had a predominant effect on T cell anergy and reduced atherosclerosis development. *In vitro*, we challenged T cells with anti-CD3 antibody or concanavalin A, and observed EGFR phosphorylation probably through transactivation. Transactivation of EGFR is well documented for G-protein-coupled receptor like ATR-1, the receptor for angiotensin II (20). This transactivation is mediated by metalloproteinase-dependent release of EGFR ligands, including EGF, TGF-α and HB-EGF, from their cell membrane-bound precursors and intermediary signaling molecules, including intracellular Ca^{2+}, Protein Kinase C, and cytosolic tyrosine kinases such as Src kinases (21).

The expression of EGFR and its ligands have been detected in experimental and human atherosclerosis (9,22). Wang et al. recently reported that EGFR engagement is important for macrophage proatherogenic activity, as its pharmacological inhibition reduced proinflammatory cytokine production, lipid uptake and oxidative stress (23). In the present study, we reported that global EGFR inhibition and $CD4^+$ T specific deletion of EGFR reduced both atherosclerosis development and progression, and induced a less inflammatory plaque phenotype.

Our findings indicate that EGFR inhibitors, widely used in patients with cancer, are unlikely to worsen the risk of cardiovascular disease, and even more suggest that EGFR may constitute a novel therapeutic target in atherosclerotic disease. The recent positive results of the CANTOS trial (24) highlighted that modulating the immune system could be a promising approach to treat atherosclerosis–related cardiovascular diseases.

Conclusions

EGFR is expressed in human and mouse $CD4^+$ T cells. EGFR pharmacological blockade or $CD4^+$ T specific invalidation induced T cell anergy and reduced both atherosclerosis development and progression in mice.

Perspectives

COMPETENCY IN MEDICAL KNOWLEDGE: EGFR is identified as a critical regulator in $CD4^+$ T cell activity both in mouse and in human. EGFR pharmacological blockade using Erlotinib or selective invalidation in $CD4^+$ T cells limited the development and the progression of experimental atherosclerosis.

TRANSLATIONAL OUTLOOK: Pharmacological inhibition of EGFR may represent a promising new immunomodulatory treatment of cardiovascular diseases preventing the progression and complications of atherosclerosis.

References

1. Onn A, Isobe T, Wu W et al. Epidermal growth factor receptor tyrosine kinase inhibitor does not improve paclitaxel effect in an orthotopic mouse model of lung cancer. Clin Cancer Res 2004;10:8613-9.

2. Gridelli C, Rossi A, Maione P et al. Erlotinib in non-small-cell lung cancer. Expert opinion on pharmacotherapy 2007;8:2579-92.

3. Guo G, Gong K, Wohlfeld B, Hatanpaa KJ, Zhao D, Habib AA. Ligand-Independent EGFR Signaling. Cancer Res 2015;75:3436-41.

4. Krakstad C, Chekenya M. Survival signalling and apoptosis resistance in glioblastomas: opportunities for targeted therapeutics. Mol Cancer 2010;9:135.

5. Bareschino MA, Schettino C, Troiani T, Martinelli E, Morgillo F, Ciardiello F. Erlotinib in cancer treatment. Annals of oncology 2007;18 Suppl 6:vi35-41.

6. Ciardiello F, Tortora G. EGFR antagonists in cancer treatment. N Engl J Med 2008;358:1160-74.

7. Chan G, Nogalski MT, Yurochko AD. Activation of EGFR on monocytes is required for human cytomegalovirus entry and mediates cellular motility. Proc Natl Acad Sci U S A 2009;106:22369-74.

8. Zaiss DM, van Loosdregt J, Gorlani A et al. Amphiregulin enhances regulatory T cell-suppressive function via the epidermal growth factor receptor. Immunity 2013;38:275-84.

9. Dreux AC, Lamb DJ, Modjtahedi H, Ferns GA. The epidermal growth factor receptors and their family of ligands: their putative role in atherogenesis. Atherosclerosis 2006;186:38-53.

10. Mallat Z, Gojova A, Sauzeau V et al. Rho-associated protein kinase contributes to early atherosclerotic lesion formation in mice. Circ Res 2003;93:884-8.

11. Morin F, Kavian N, Marut W et al. Inhibition of EGFR Tyrosine Kinase by Erlotinib Prevents Sclerodermatous Graft-Versus-Host Disease in a Mouse Model. J Invest Dermatol 2015;135:2385-93.

12. Dudu V, Able RA, Jr., Rotari V, Kong Q, Vazquez M. Role of Epidermal Growth Factor-Triggered PI3K/Akt Signaling in the Migration of Medulloblastoma-Derived Cells. Cell Mol Bioeng 2012;5:502-413.

13. Camorani S, Crescenzi E, Colecchia D et al. Aptamer targeting EGFRvIII mutant hampers its constitutive autophosphorylation and affects migration, invasion and proliferation of glioblastoma cells. Oncotarget 2015;6:37570-87.

14. Tai YL, Chu PY, Lai IR et al. An EGFR/Src-dependent beta4 integrin/FAK complex contributes to malignancy of breast cancer. Sci Rep 2015;5:16408.

15. Luo Q, Gu Y, Zheng W et al. Erlotinib inhibits T-cell-mediated immune response via down-regulation of the c-Raf/ERK cascade and Akt signaling pathway. Toxicology and applied pharmacology 2011;251:130-6.

16. Bryant JA, Finn RS, Slamon DJ, Cloughesy TF, Charles AC. EGF activates intracellular and intercellular calcium signaling by distinct pathways in tumor cells. Cancer Biol Ther 2004;3:1243-9.

17. Subramanian M, Thorp E, Hansson GK, Tabas I. Treg-mediated suppression of atherosclerosis requires MYD88 signaling in DCs. J Clin Invest 2013;123:179-88.

18. Ait-Oufella H, Sage AP, Mallat Z, Tedgui A. Adaptive (T and B cells) immunity and control by dendritic cells in atherosclerosis. Circ Res 2014;114:1640-60.

19. Ait-Oufella H, Salomon BL, Potteaux S et al. Natural regulatory T cells control the development of atherosclerosis in mice. Nat Med 2006;12:178-80.

20. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. Am J Physiol Cell Physiol 2007;292:C82-97.

21. Higuchi S, Ohtsu H, Suzuki H, Shirai H, Frank GD, Eguchi S. Angiotensin II signal transduction through the AT1 receptor: novel insights into mechanisms and pathophysiology. Clin Sci (Lond) 2007;112:417-28.

22. Nakata A, Miyagawa J, Yamashita S et al. Localization of heparin-binding epidermal growth factor-like growth factor in human coronary arteries. Possible roles of HB-EGF in the formation of coronary atherosclerosis. Circulation 1996;94:2778-86.

23. Wang L, Huang Z, Huang W et al. Inhibition of epidermal growth factor receptor attenuates atherosclerosis via decreasing inflammation and oxidative stress. Sci Rep 2017;8:45917.

24. Ridker PM, Everett BM, Thuren T et al. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. N Engl J Med. 2017 ;377:1199-1131.

Figure Legends

Central Illustration: EGFR blockade limits pro-atherogenic activity of CD4+ T Cells. EGFR engagement orchestrates CD4+T cell proliferation, cytokine production and recruitment within atherosclerotic plaques. Pharmalogical blockade of EGFR signaling may constitute an attractive novel approach for the treatment of atherosclerosis.

Figure 1: **EGFR receptor expression and activation in mouse T cells**. A, EGFR is expressed by splenocytes (green) and co-localizes with $CD4^+$ T cells (Red). B, EGFR is expressed within mouse atherosclerotic lesions (Red) and co-localizes with $CD4^+$ T cells (green). Staining was performed on atherosclerotic plaques (aortic sinus) from *Ldlr*$^{-/-}$ mouse fed a high fat diet during 6 weeks. C, stimulation of purified $CD4^+$ T cells by coated anti-CD3 during 60 minutes induced focal EGFR phosphorylation that was abolished by AG-1478 (1 μM). D, Flow cytometry quantification of Erk phosphorylation gated on $CD3^+CD4^+$ T cells after 30 minutes of anti-CD3 (5μg/ml) or Concanavalin A (10 μg/ml) stimulation (N=4/group). E, Naive CD4+ T cells were stimulated with α-CD3/CD28 antibodies for 72h in the presence of 1μM or 10μM AG1478 before mesuring intracellular free calcium concentration (Fura-2-AM fluorescent probe). Each trace is the fluorescence mean (340/380nm) of three independant experiences (10<N<40 cells per experience). Slope factors are calculated between 120 and 240 seconds for each experimental condition. *, P <0.05, ** P<0.01 *** and P<0.001. Data are expressed as median and percentiles (5^{th}-95^{th}).

Figure 2. Effects of EGFR pharmacological inhibition on mouse T cell functions. A, *in vitro* AG-1478 induced a dose-dependent reduction of $CD4^+$ T cell proliferation in response to soluble anti-CD3 stimulation, in the presence of $CD11c^+$ dendritic cells. B, *in vitro* AG-1478 induced a dose-dependent reduction of OT-II $CD4^+$ T cell proliferation after OVA stimulation in the

presence of $CD11c^+$ dendritic cells. C, effects of different concentrations of AG-1478 on T cell apoptosis, defined as annexin V^{pos} $7\text{-}AAD^{neg}$ cells. Purified $CD4^+$ T cells were stimulated by coated anti-CD3 (5μg/ml) or Concanavalin A (10 μg/ml) and cytokine production was evaluated by flow cytometry (D, E) or by ELISA in the supernatants (F, G, H, I). Data are expressed as median and percentiles (5^{th}-95^{th}).

Figure 3. EGFR pharmacological inhibition limited T cell activation *in vivo* and reduced atherosclerosis. A, protocol of pharmacological blockade of EGFR in male *Ldlr*$^{-/-}$ mice (Erlotinib 15mg/kg/day, orally) (N=8-9/group). Cholesterolemia (B) and splenocyte number (C) at sacrifice. Proliferation (D) and cytokine production (ELISA, E) of purified splenic $CD4^+$ T cells from control of erlotinib-treated animals after 48 hours of coated anti-CD3 stimulation. F, Quantification and representative photomicrographs of T cell infiltration in atherosclerotic lesions from *Ldlr*$^{-/-}$ animals treated by PBS or erlotinib. N=8-9/group. G, Quantification and representative photomicrographs of atherosclerotic lesion size in the aortic sinus from *Ldlr*$^{-/-}$ animals treated by PBS or Erlotinib. N=8-9/group. H, protocol of pharmacological blockade of EGFR in *Ldlr*$^{-/-}$ female mice after an 8-week period of high fat diet, (Erlotinib 15mg/kg/day, orally) (N=7/group). I, representative photomicrographs and quantification of atherosclerotic lesion size along the thoracic aorta (%) from *Ldlr*$^{-/-}$ animals orally treated by PBS or Erlotinib. * $P<0.05$, **, $P<0.01$. Data are expressed as median and percentiles (5^{th}-95^{th}). Adv, Adventitia.

Figure 4: EGFR expression and functions in human T cells. A, Blood human $CD4^+$ T cells were purified and stimulated with Concanavalin (10 μg/ml) during 24 hours. EGFR is expressed $CD4^+$ T cells after activation (fluorescent staining, Green). EGFR inhibition using tyrosine Kinase inhibitor (B) or neutralizing antibody (C) decreased T cell proliferation after 48 hours of culture at different concentrations of CD3/CD28-coated beads. Proliferation was measured by

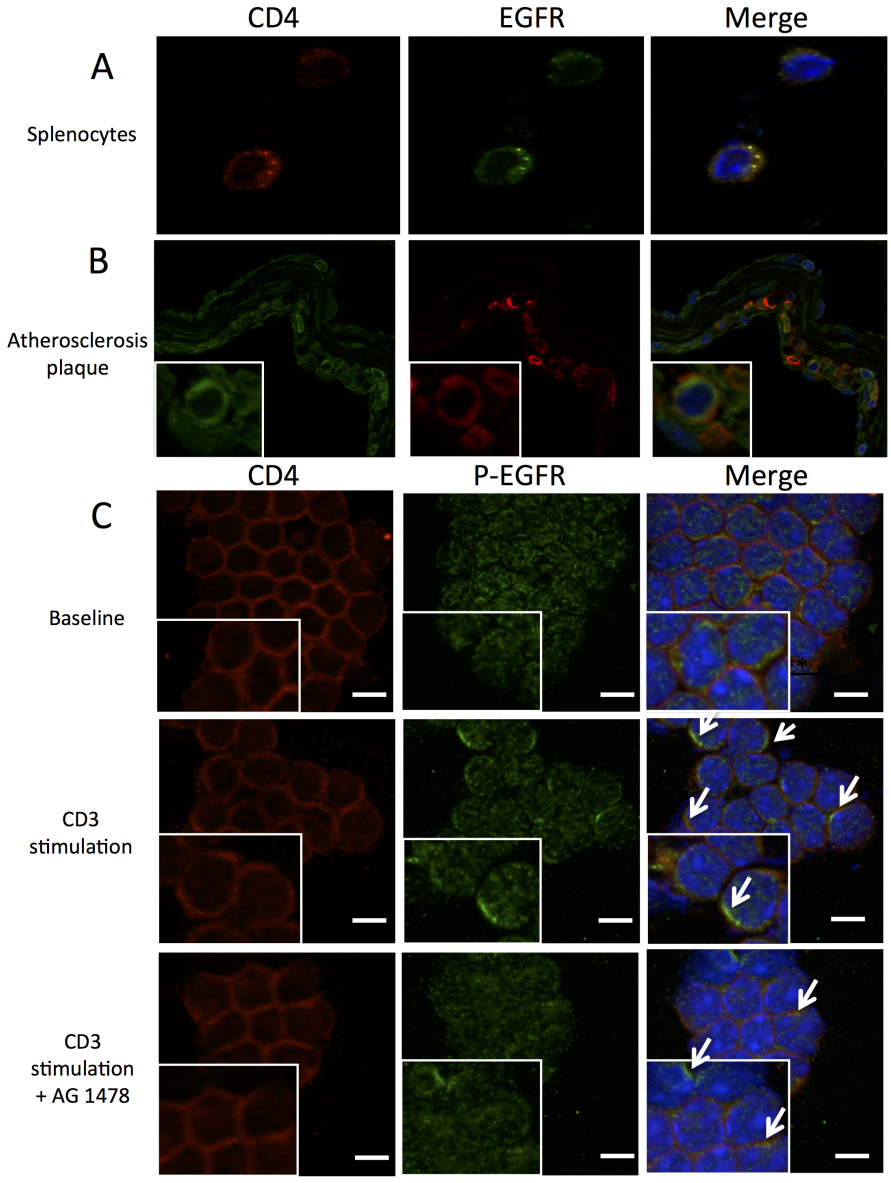
incorporation of methyl-$^{[3H]}$thymidine (N=4). (D) Pharmacological inhibition of EGFR using erlotinib, an EGFR TKI, blocked in vivo T cell proliferation. T cells were isolated from 3 patients with lung cancer just before and one month after initiation of erlotinib treatment. Cells were stimulated with a ratio of 2 beads for 1 cell and proliferation measured by methyl-$^{[3H]}$thymidine incorporation. *p<0.05. Bar scale 10 μm. Data are expressed as median and percentiles (5^{th}-95^{th}). TKI, Tyrosine Kinase inhibitor.

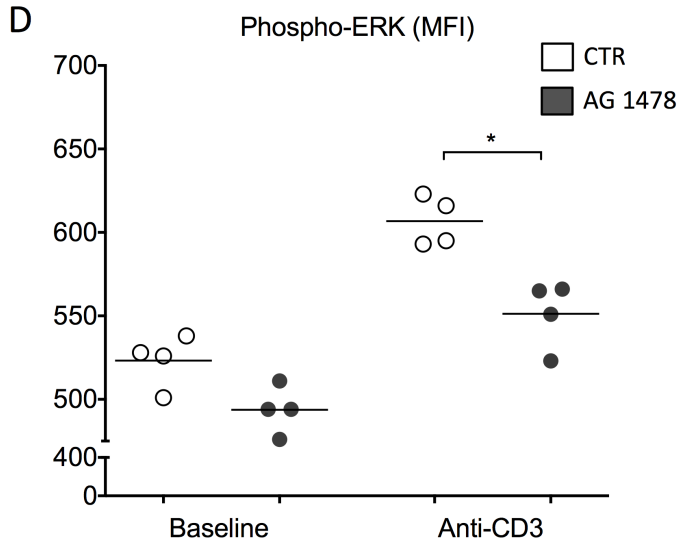
Figure 5. Selective EGFR deletion in CD4$^+$ T cells induced anergy. A, *In vitro,* purified CD4$^+$ T cells from control *Cd4-Cre/Egf-r*$^{+/+}$ (white) or *Cd4-Cre/Egf-r*$^{lox/lox}$ mice (black) were stimulated by soluble anti-CD3 with or without co-incubation with CD11c$^+$ dendritic cells (APC). Genetic invalidation of *Egfr* reduced T cell proliferation (A), IFN-γ (B) and IL-4 (C) production but had no effect on IL-10 (D) (ELISA in the supernatant). E, Representative examples and quantitative analysis of intracellular IFN-γ staining of isolated splenocytes from control *Cd4-Cre/Egf-r*$^{+/+}$ (white) or *Cd4-Cre/Egf-r*$^{lox/lox}$ *mice*. Plots are gated on CD4$^+$ and CD8$^+$ T cells. Numbers in each quadrant indicate percentages of cells. F, representative examples of CD4$^+$ T cell *in vivo* proliferation. *Cd4-Cre/Egfr*$^{+/+}$ and *Cd4-Cre/Egfr*$^{lox/lox}$ purified CD4$^+$ T cells from pooled spleens and lymph nodes were labeled with CFSE fluorescent dye. Apoe$^{-/-}$/*Rag2*$^{-/-}$ mice received 20.10^6 CD4$^+$ T cell–depleted splenocytes resupplemented with 8.10^6 purified CD4$^+$ T cells from either *Cd4-Cre/Egfr*$^{+/+}$ or *Cd4-CreEgfr*$^{lox/lox}$ animals. The proliferation of adoptively transferred cells was visualized by flow cytometric analysis of CFSE-labeled CD4$^+$ T cells at day 10 after transfer. N=5/group, * P<0.05, **P<0.01. Data are expressed as median and percentiles (5^{th}-95^{th}). APC, Antigen Presenting Cell.

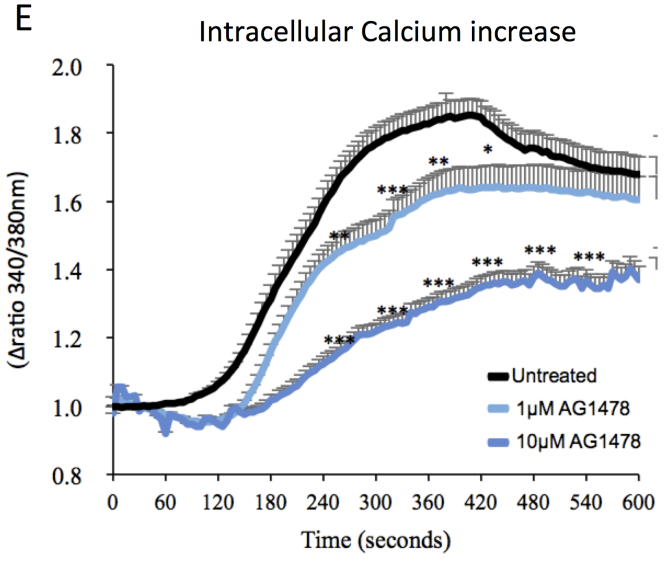
Figure 6. **Selective EGFR deletion in CD4$^+$ T cells in chimeric *Ldlr*$^{-/-}$ mouse model of atherosclerosis.** A, representative examples and FACS quantification of CD4$^+$ T subset. B,

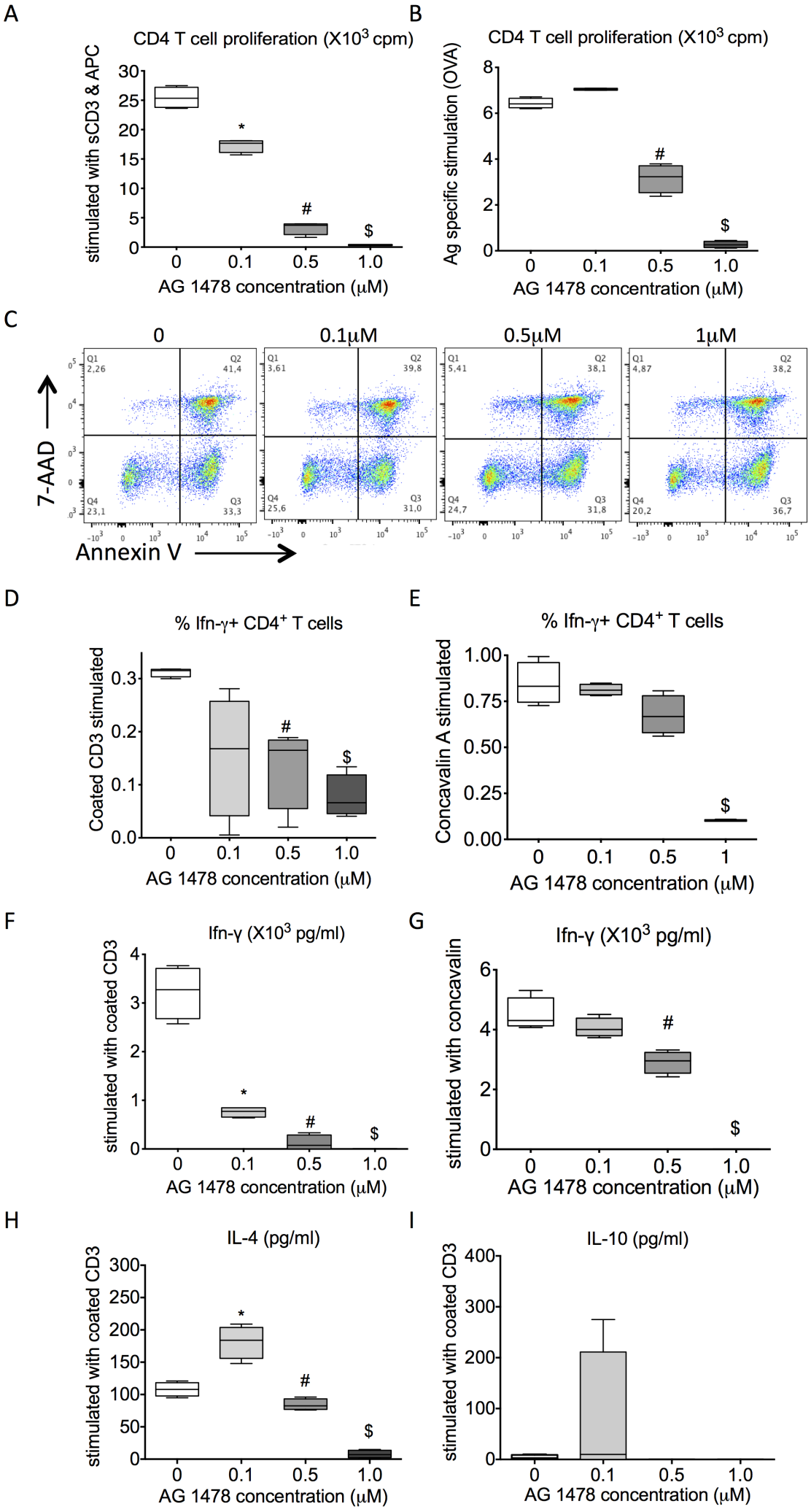
representative examples and FACS quantification of $CD4^+CD25^+FoxP3^+$ regulatory T cells. Representative examples and quantification of CD69 expression by $CD4^+$ T cell (C) and $CD44^{high}$ expression by $CD62L^-CD4^+$ T cells by flow cytometry (D). E, *In vitro* suppressive tests of effector $CD25^-$ T cell proliferation by co-culture with $CD4^+CD25^{high}$ regulatory T cells isolated from chimeric *Cd4-Cre/Egfr*$^{+/+}$/*Ldlr*$^{-/-}$ or chimeric *Cd4-Cre/Egf-r*$^{lox/lox}$/*Ldlr*$^{-/-}$ mice. Purified $CD4^+$ T cells from *chimeric* mice were stimulated by soluble anti-CD3. Genetic invalidation of *Egfr* reduced T cell proliferation (F), IFN-γ and IL-4 production but had no effect on IL-10 (G, ELISA in the supernatant). N=8-9/group, *, P<0.05. Data are expressed as median and percentiles (5^{th}-95^{th}).

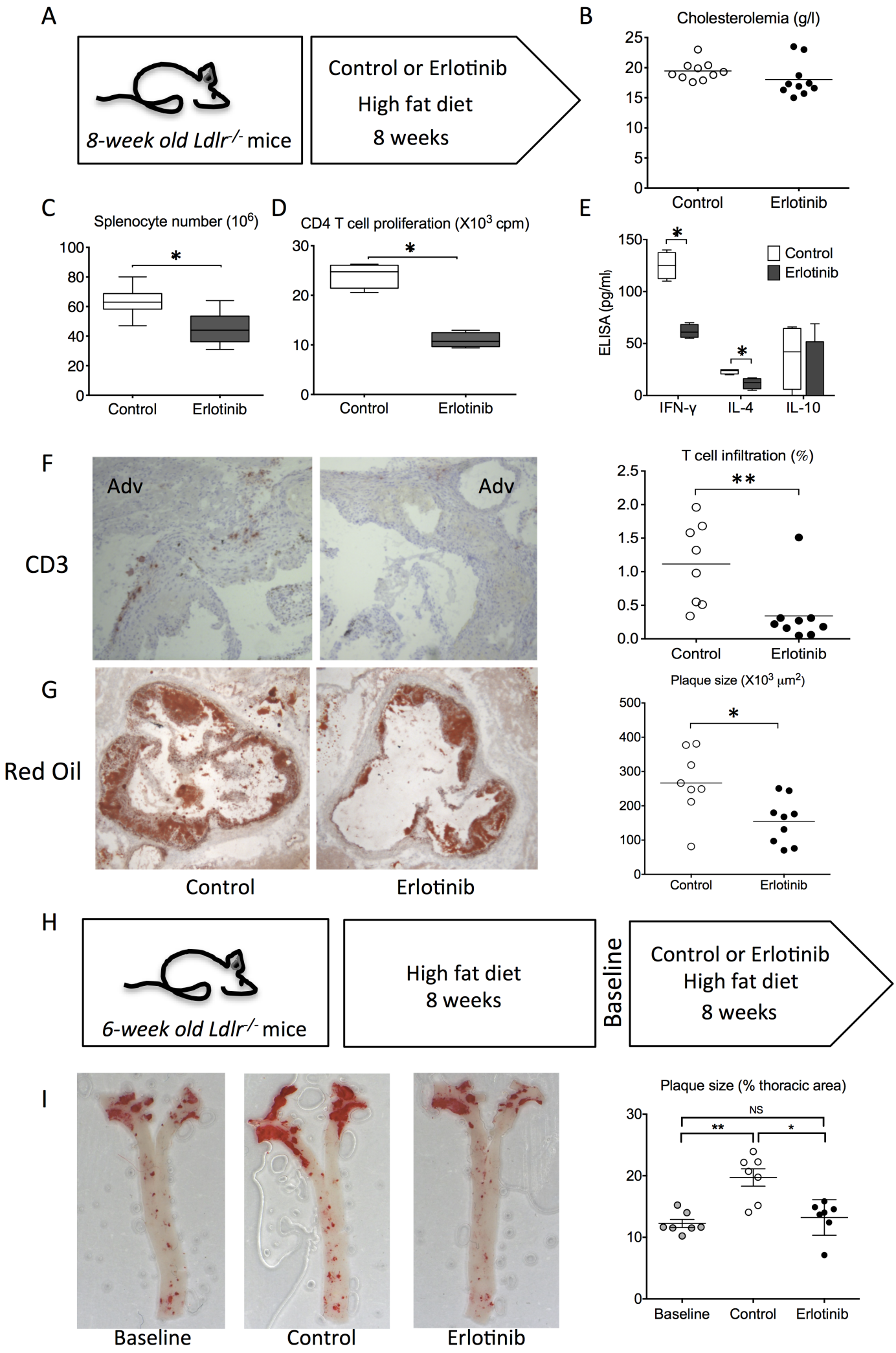
Figure 7. Selective EGFR deletion in $CD4^+$ T cells reduced experimental atherosclerosis development. A, Representative photomicrographs of Oil Red staining and quantitative analysis of atherosclerotic lesion size in the aortic root of irradiated *Ldlr*$^{-/-}$ mice reconstituted with bone marrow from either *Cd4-Cre/Egfr*$^{+/+}$ or *Cd4-Cre/Egfr*$^{lox/lox}$ mice and put on a high fat diet for 4 (N=5-6/group), 6 (N=9/group) and 12 weeks (N=9-10/group). B, Representative photomicrographs and quantitative analysis of T cells (CD3 staining) in atherosclerotic lesion of irradiated *Ldlr*$^{-/-}$ mice reconstituted with bone marrow from either *Cd4-Cre/Egfr*$^{+/+}$ or *Cd4-Cre/Egfr*$^{lox/lox}$ mice and put on a high fat diet during 6 weeks. * P<0.05.

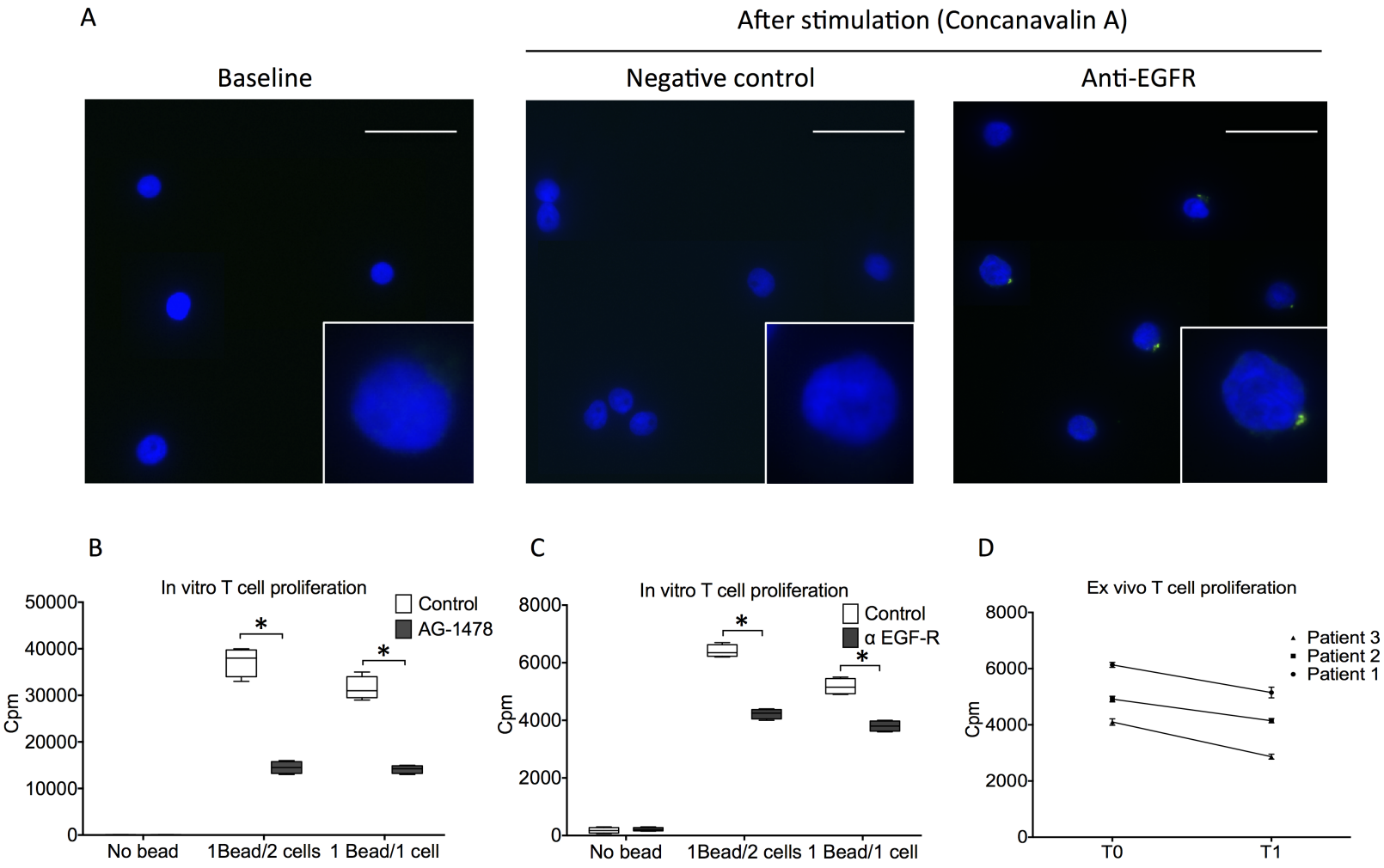

CD4
EGFR
Merge
A
Splenocytes
B
Atherosclerosis plaque
CD4
P-EGFR
Merge
C
Baseline
CD3 stimulation
CD3 stimulation + AG 1478

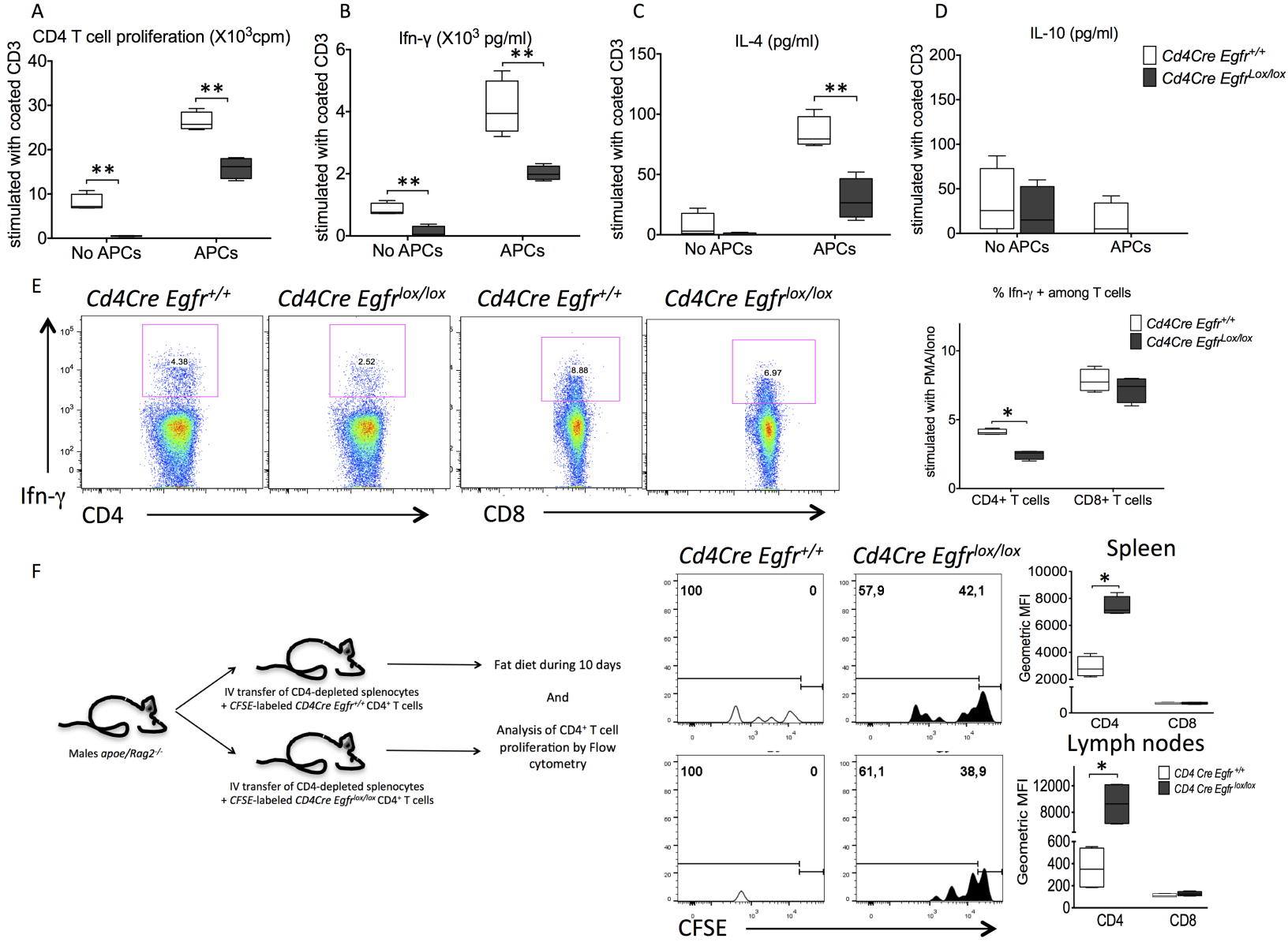

D
Phospho-ERK (MFI)
CTR
AG 1478
700
650
600
550
500
400
0
*
Baseline
Anti-CD3

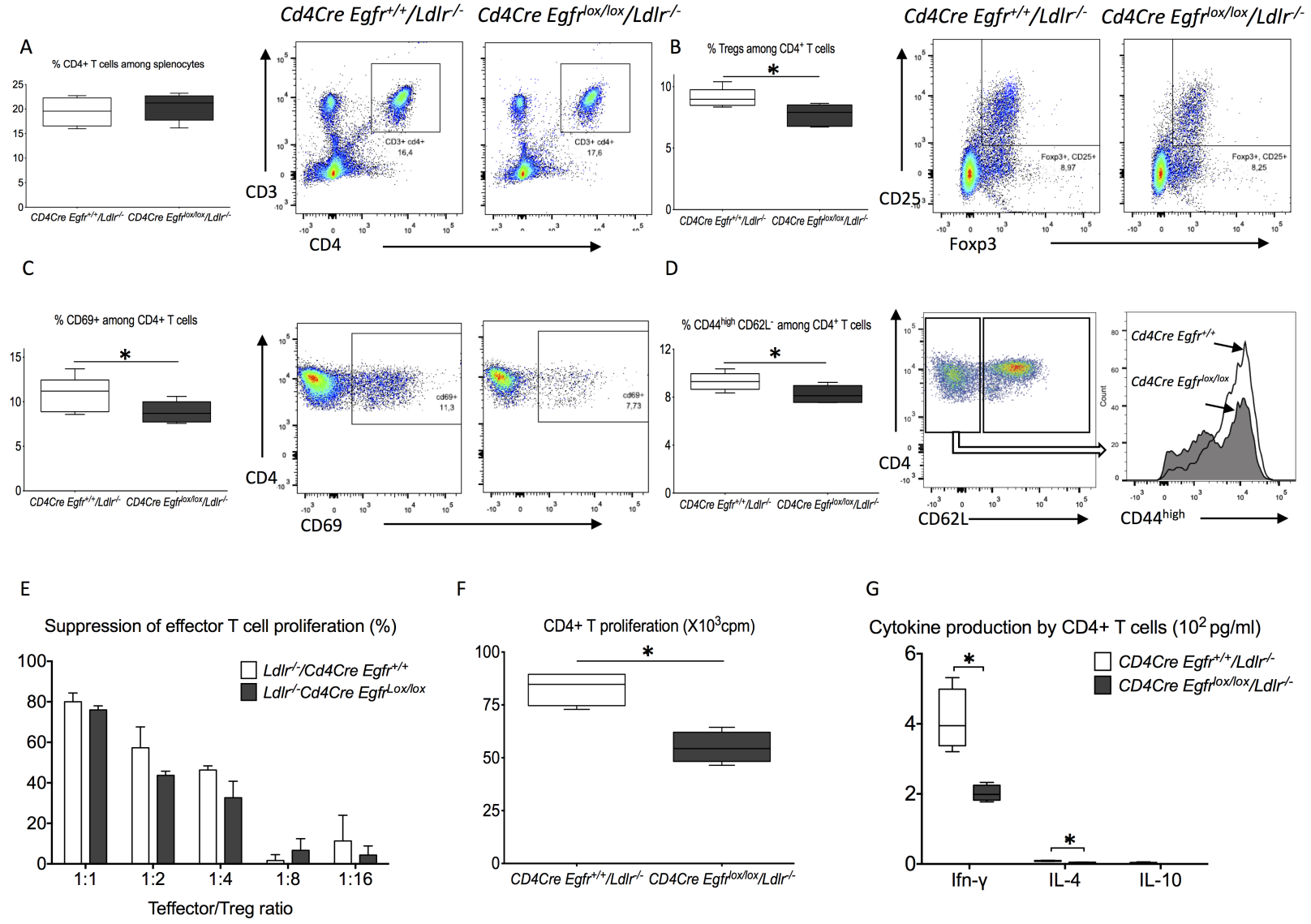

E
Intracellular Calcium increase
(Δratio 340/380nm)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
60
120
180
240
300
360
420
480
540
600
Time (seconds)
Untreated
1μM AG1478
10μM AG1478


A
CD4 T cell proliferation (X10³ cpm)
stimulated with sCD3 & APC
AG 1478 concentration (μM)
B
CD4 T cell proliferation (X10³ cpm)
Ag specific stimulation (OVA)
AG 1478 concentration (μM)
C
0
0.1μM
0.5μM
1μM
7-AAD
Annexin V
D
% Ifn-γ+ CD4⁺ T cells
Coated CD3 stimulated
AG 1478 concentration (μM)
E
% Ifn-γ+ CD4⁺ T cells
Concavalin A stimulated
AG 1478 concentration (μM)
F
Ifn-γ (X10³ pg/ml)
stimulated with coated CD3
AG 1478 concentration (μM)
G
Ifn-γ (X10³ pg/ml)
stimulated with concavalin
AG 1478 concentration (μM)
H
IL-4 (pg/ml)
stimulated with coated CD3
AG 1478 concentration (μM)
I
IL-10 (pg/ml)
stimulated with coated CD3
AG 1478 concentration (μM)


A
8-week old Ldlr-/- mice
Control or Erlotinib
High fat diet
8 weeks
B
Cholesterolemia (g/l)
25
20
15
10
5
0
Control
Erlotinib
C
Splenocyte number (10^6)
100
80
60
40
20
0
*
Control
Erlotinib
D
CD4 T cell proliferation (X10^3 cpm)
30
20
10
0
*
Control
Erlotinib
E
ELISA (pg/ml)
150
100
50
0
*
*
Control
Erlotinib
IFN-γ
IL-4
IL-10
F
CD3
Adv
Adv
T cell infiltration (%)
2.5
2.0
1.5
1.0
0.5
0.0
**
Control
Erlotinib
G
Red Oil
Control
Erlotinib
Plaque size (X10^3 μm^2)
500
400
300
200
100
0
*
Control
Erlotinib
H
6-week old Ldlr-/- mice
High fat diet
8 weeks
Baseline
Control or Erlotinib
High fat diet
8 weeks
I
Baseline
Control
Erlotinib
Plaque size (% thoracic area)
30
20
10
0
NS
**
*
Baseline
Control
Erlotinib


A
After stimulation (Concanavalin A)
Baseline
Negative control
Anti-EGFR
B
In vitro T cell proliferation
Control
AG-1478
Cpm
50000
40000
30000
20000
10000
0
No bead
1Bead/2 cells
1 Bead/1 cell
C
In vitro T cell proliferation
Control
α EGF-R
Cpm
8000
6000
4000
2000
0
No bead
1Bead/2 cells
1 Bead/1 cell
D
Ex vivo T cell proliferation
Patient 3
Patient 2
Patient 1
Cpm
8000
6000
4000
2000
0
T0
T1


A
CD4 T cell proliferation (X10³cpm)
stimulated with coated CD3
No APCs
APCs
B
Ifn-γ (X10³ pg/ml)
C
IL-4 (pg/ml)
D
IL-10 (pg/ml)
Cd4Cre Egfr+/+
Cd4Cre EgfrLox/lox
E
Cd4Cre Egfr+/+
Cd4Cre Egfrlox/lox
4.38
2.52
8.88
6.97
Ifn-γ
CD4
CD8
% Ifn-γ + among T cells
stimulated with PMA/Iono
CD4+ T cells
CD8+ T cells
F
Males apoe/Rag2-/-
IV transfer of CD4-depleted splenocytes
+ CFSE-labeled CD4Cre Egfr+/+ CD4+ T cells
IV transfer of CD4-depleted splenocytes
+ CFSE-labeled CD4Cre Egfrlox/lox CD4+ T cells
Fat diet during 10 days
And
Analysis of CD4+ T cell proliferation by Flow cytometry
100
0
57,9
42,1
61,1
38,9
CFSE
Spleen
Geometric MFI
Lymph nodes
CD4 Cre Egfr +/+
CD4 Cre Egfr lox/lox

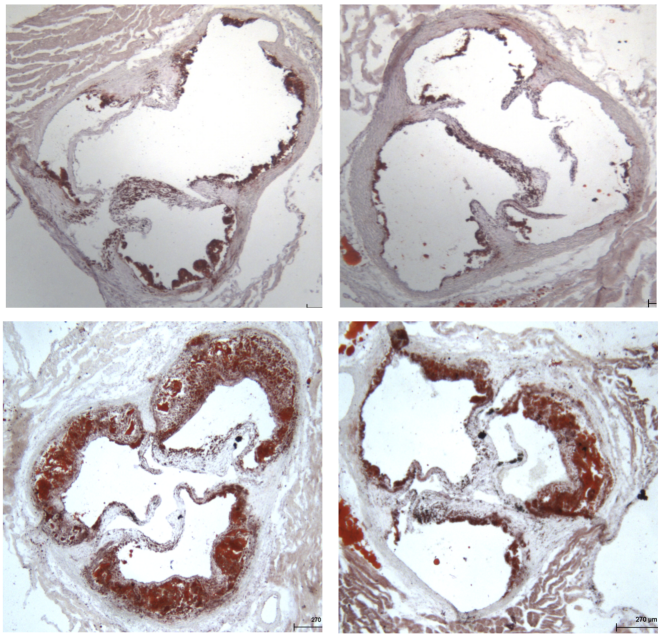

A
% CD4+ T cells among splenocytes
CD4Cre Egfr+/+/Ldlr-/- CD4Cre Egfrlox/lox/Ldlr-/-
Cd4Cre Egfr+/+/Ldlr-/- Cd4Cre Egfrlox/lox/Ldlr-/-
CD3+ cd4+ 16,4
CD3+ cd4+ 17,6
CD3
CD4
B
% Tregs among CD4+ T cells
Cd4Cre Egfr+/+/Ldlr-/- Cd4Cre Egfrlox/lox/Ldlr-/-
Foxp3+, CD25+ 8,97
Foxp3+, CD25+ 8,25
CD25
Foxp3
C
% CD69+ among CD4+ T cells
CD4Cre Egfr+/+/Ldlr-/- CD4Cre Egfrlox/lox/Ldlr-/-
cd69+ 11,3
cd69+ 7,73
CD4
CD69
D
% CD44high CD62L- among CD4+ T cells
CD4Cre Egfr+/+/Ldlr-/- CD4Cre Egfrlox/lox/Ldlr-/-
CD4
CD62L
Cd4Cre Egfr+/+
Cd4Cre Egfrlox/lox
Count
CD44high
E
Suppression of effector T cell proliferation (%)
Ldlr-/-/Cd4Cre Egfr+/+
Ldlr-/-Cd4Cre EgfrLox/lox
1:1
1:2
1:4
1:8
1:16
Teffector/Treg ratio
F
CD4+ T proliferation (X10³cpm)
CD4Cre Egfr+/+/Ldlr-/- CD4Cre Egfrlox/lox/Ldlr-/-
G
Cytokine production by CD4+ T cells (10² pg/ml)
CD4Cre Egfr+/+/Ldlr-/-
CD4Cre Egfrlox/lox/Ldlr-/-
Ifn-γ
IL-4
IL-10

A

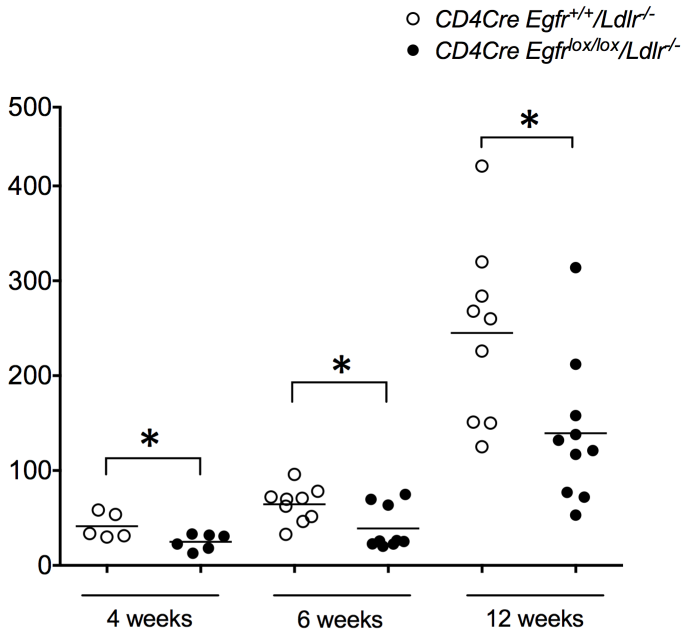
Cd4Cre Egfr$^{+/+}$/Ldlr$^{-/-}$ *Cd4Cre Egfr$^{Lox/Lox}$/Ldlr$^{-/-}$*

4 weeks of fat diet

12 weeks of fat diet



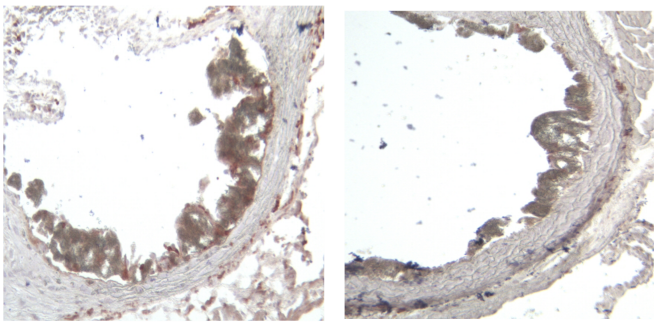
Plaque size (Aortic sinus, X10^{3}μm^2)



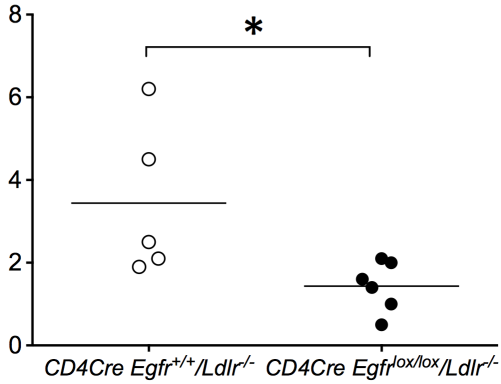
B

Cd4Cre Egfr$^{+/+}$/Ldlr$^{-/-}$ *Cd4Cre Egfr$^{Lox/Lox}$/Ldlr$^{-/-}$*

Anti-CD3 staining



T cell infiltration (% plaque area)



Online Appendix- Methods

Cell culture and proliferation assays

Cells were cultured in RPMI 1640 supplemented with Glutamax, 10% FCS, 0.02 mM β-mercapto-ethanol and antibiotics. To analyze T cell proliferation, $1x10^5$ $CD4^+$ cells/well were co-cultured with 10^4 $CD11c^+$ dendritic cells and soluble anti-CD3 (1μg/ml) for 72 h. One μCi [^{3}H] thymidine was added to each well during the last 16 h. Cells were cultured at 37°C for 72 h and pulsed with 1 μCi [^{3}H] thymidine (Amersham) for the last 18 h. Thymidine incorporation was assessed using a TopCount NXT scintillation counter (Perkin Elmer). To analyze regulatory T cell functions, effectors T cells were cultured alone or co-cultured with purified regulatory T cells (at 1:8, 1:4, 1:2 or 1:1 ratio) in bottom-rounded 96-well microplates ($0.5x10^5$ cells/well; total volume 200 μl/well). Cells were stimulated with purified soluble CD3-specific antibody (1 μg/ml, Pharmingen) in the presence of antigen-presenting cells purified on CD11c-coated magnetic beads (Miltenyi Biotech). Cells were cultured at 37°C for 72 h and pulsed with 1 μCi [^{3}H] thymidine (Amersham) for the last 18 h. Thymidine incorporation was assessed using a TopCount NXT scintillation counter (Perkin Elmer).

To explore *in vivo* proliferation, *Cd4Cre Egfr*$^{+/+}$ and *Cd4Cre Egfr*$^{lox/lox}$ purified $CD4^+$ T cells from pooled spleens and lymph nodes were labeled with CFSE fluorescent dye (5uM, VybrantCFDASE; Invitrogen–Molecular Probes) according to the instructions of the manufacturer. *Apoe*$^{-/-}$*/Rag2*$^{-/-}$ mice received $20x10^6$ $CD4^+$ T cell–depleted splenocytes resupplemented with $8x10^6$ purified $CD4^+$ T cells from either *Cd4Cre Egfr*$^{+/+}$ or *Cd4Cre Egfr*$^{lox/lox}$ mice. The proliferation of adoptively transferred cells was visualized by flow cytometric analysis of CFSE-labeled $CD4^+$ T cells at day 7 after transfer.

Human lymphocytes were isolated from blood (negative selection, Miltenyi Biotech). Blood samples from patients (with non-small lung cell cancer) and healthy volunteers was collected after informed consent, as part of the OncoHEGP clinical protocol at Georges Pompidou European Hospital, approved by the institutional ethics committee (CPP Ile de France II n°2012-08-09). Venous blood was centrifuged with a Histopaque™ gradient (density: 1.077 g/cm^3, 30 min, 500g, 20 °C; Sigma-Aldrich, Saint Louis, US). Cells were washed twice with warm PBS and cell viability, as well as concentration, was determined using the trypan blue exclusion test. Cells were cultured in the same medium as that used for mouse cells. Specific T cell proliferation was induced using CD3/CD28-coated beads (Invitrogen™, Life Technologies, Paisley, UK) with different Bead/T cell ratio. EGFR engagement was blocked using the specific pharmacological inhibitor AG-1478 (Calbiochem™, Merck Millipore, Darmstadt, Germany). We also used the specific EGFR mAb, Erbitux (Cetuximab®, Merk Sorono, 1µg/mL), which links the EGFR extracellular domain and prevents binding of ligands.

Cytosolic calcium recording

Intracellular Ca2+ activity was determined as previously described (1). Briefly, CD4+ T cells were loaded at 1x10^6/mL with Fura-2-AM (5µM, with 0.015% Pluronic F127 in RPMI medium) (Molecular Probes, ThermoFisher Scientific) for 30 min at room temperature, washed and then resuspended in RPMI medium. Cells were attached to poly(L)-lysine-coated petri dish (Sigma-Aldrich) for 20 min at 37°C and Fura-2 dual excitation was accomplished using 340-380nm excitation filters at 5 seconds intervals. Cells were then perfused with the bath solution in the presence or absence of 2 mM extracellular Ca2+ and stimulated with 5µg/mL of anti-CD3 antibody (BD biosciences). Variation of fluorescence ratio reveal intracellular variation of free

cytosolic calcium concentration. Data were analyzed using Leica DMI 6000 inverted microscope using MetaFluor Software (Leica, Wetzlar, Germany).

Western Blot

After extraction from isolated $CD4^+$ T cells with RIPA buffer, proteins were quantified using a BCA protein assay kit (iNtRON Biotechnology). Equal amounts of proteins were loaded onto sodium dodecyl sulfate-polyacrylamide electrophoresis gels (4%-12% Bis-Tris Criterion XT gels, Biorad) for separation and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with milk and probed with different antibodies: anti-Akt (Cell Signaling Technology, C67E7), anti-Phospho Akt (Cell Signaling Technology, D9E), anti-ERK1/2 (Cell Signaling Technology, 137F5), anti-Phospho ERK T202/Y204 (Cell Signaling Technology, D13.14.4E) and anti-TUBA/a-tubulin (Abcam, ab6160). Protein loading was monitored using an anti-tubulin antibody (Abcam, Inc.). The membranes were then probed with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, 7074 and 7076) and the bands were visualized by enhanced chemiluminescence (Clarity Western ECL substrate; Bio-Rad, 170–5061). Antigens were revealed by enhanced chemiluminescence (Supersignal West Pico; Pierce) and detected on a LAS-4000 imaging system (Fuji, LAS4000, Burlington, NJ, USA). Densitometric analysis with ImageJ software was used for quantification.

Flow cytometry

Cells were labeled with APC-conjugated anti-CD3ε (145-2C11), FITC- or PE-Cy7-conjugated anti-CD4 (RM4-5), APC-conjugated anti-CD25 (PC61.5), PE-Cy7-conjugated anti-CD11b (M1/70), PE-conjugated anti-Foxp3 (PFJK-16s), eFluor 450-conjugated anti-CD62L (MEL-14) and APC-conjugated anti-CD44 (IM7) mAbs from eBioscience. PE-conjugated anti-Ly6G (1A8), Alexa Fluor 488-conjugated anti-CD11b (M1/70), APC-conjugated F4/80 (BM8)

FITC-conjugated anti-CD19 (1D3), Alexa Fluor 700-conjugated anti-CD8 (53-6.7), APC-conjugated anti-IFNγ (XMG1.2), PE-conjugated anti-IL10 (JES-16E3), Alexa Fluor 647-conjugated anti-ERK1/2 (pT202/pY204) were from BD Biosciences. Alexa fluor 647-conjugated anti-7/4 was from Serotec. For blood staining, erythrocytes were lyzed using BD FACS lysis solution (BD Biosciences). For intracellular cytokine staining, lymphocytes were stimulated in vitro with leukocyte activation cocktail (BD Biosciences) according to manufacturer's instructions for 4 h. Surface staining was performed before permeabilization using Foxp3 staining buffer kit (eBioscience) and intracellular staining. Forward scatter (FSC) and side scatter (SSC) were used to gate live cells excluding RBC, debris, and cell aggregates in total splenocytes. Cells were analyzed using a BD CantoII or BD LSRII flow cytometer (BD Biosciences).

Apoptosis susceptibility of purified $CD4^+$ T cells was determined using the apoptosis detection kit Annexin V- (FITC) and 7AAD (APC) (BD Biosciences, San Jose, CA, US) according to the manufacturer's instructions.

References

1. Di L, Srivastava S, Zhdanova O et al. Inhibition of the K+ channel KCa3.1 ameliorates T cell-mediated colitis. Proc Natl Acad Sci U S A 2010;107:1541-6.

Online Figure Legends

Online Figure 1. EGFR pharmacological inhibition reduced ERK phosphorylation

Online Figure 2. No effect of EGFR pharmacological inhibition on CD4+ T cell morphology.

Online Figure 3. EGFR pharmacological inhibition reduced IL-2 production by CD4+ T cells.

Online Figure 4. Erlotinib treatment had no effect on animal weight but induced a reduction of spleen size.

Online Figure 5. Erlotinib treatment reduced CD4+ T cell activation

Online Figure 6. Erlotinib treatment blocked atherosclerosis progression.

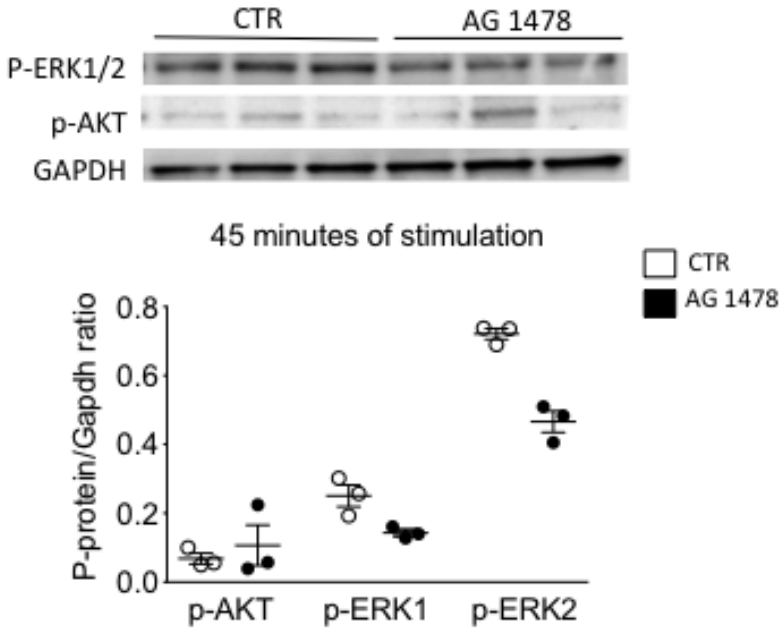
Online Figure 7. Absence of EGFR expression on CD4+ T cells from Cd4 Cre EgfrLox/lox mice.

Online Figure 8. EGFR deficiency reduced CD4+ T cell apoptosis.

Online Figure 9. EGFR deficiency had no impact on cholesterolemia.

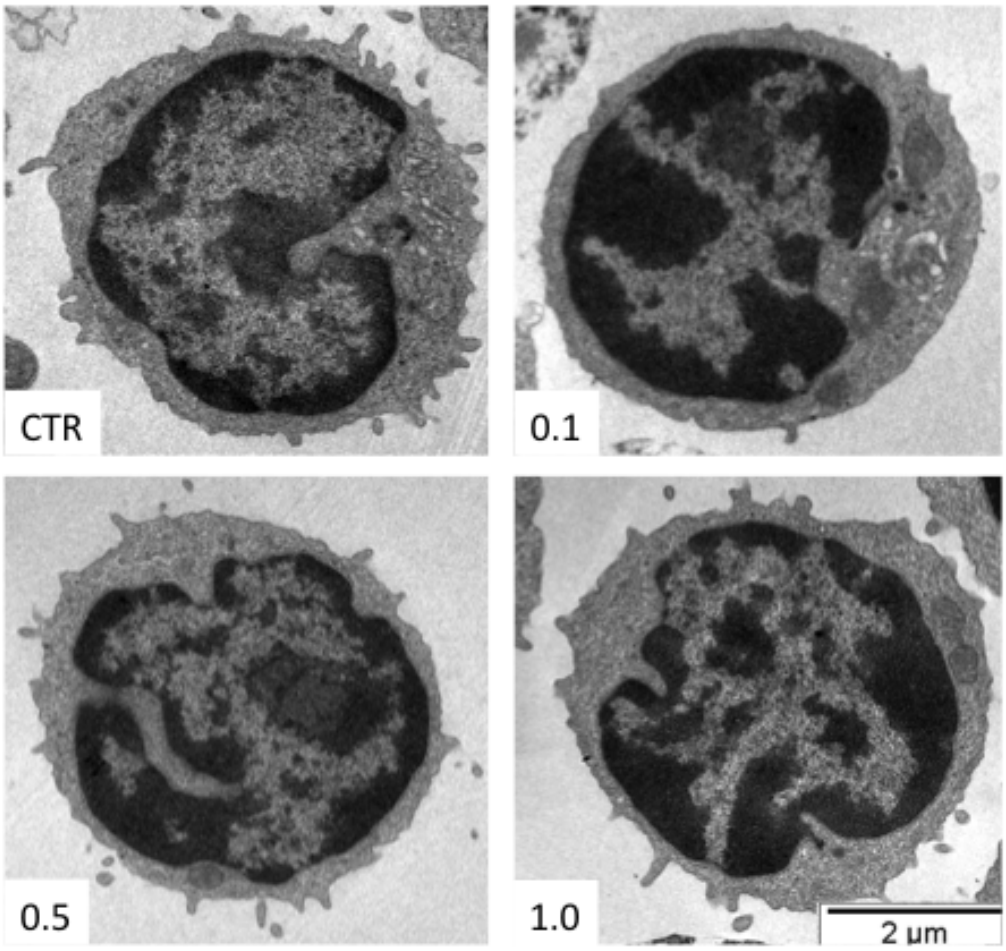
Online Figure 10. EGFR deficiency in CD4+ T cells had no impact on macrophage content.

Supplemental figure 1



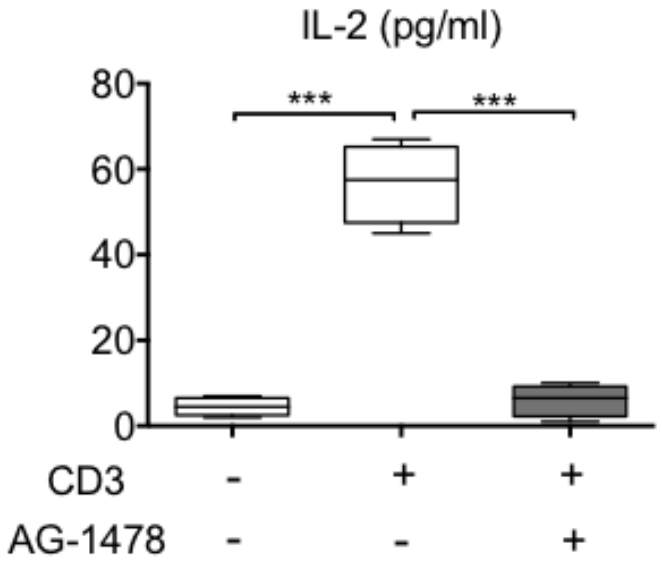
EGFR pharmacological inhibition using AG 1478 (1 μM) reduced ERK phosphorylation. Western blot in purified splenic $CD4^+$ T cells after 45 minutes of anti-CD3 stimulation.

Supplemental figure 2



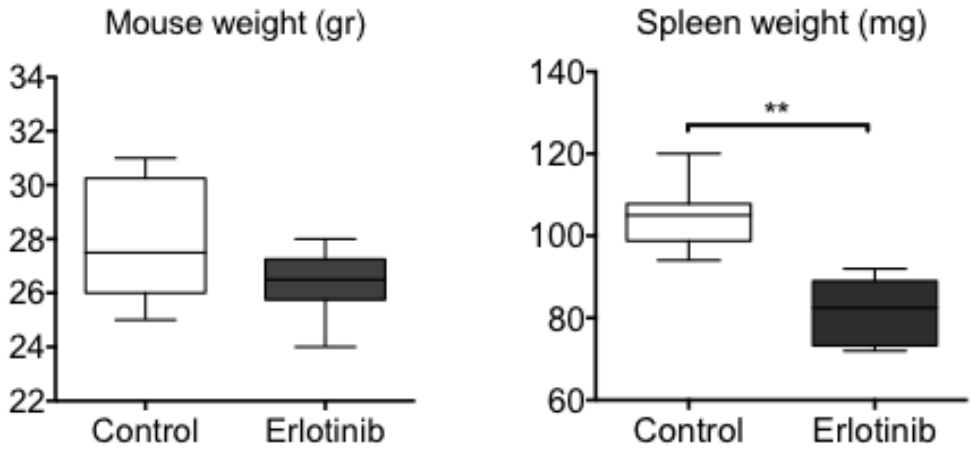
Morphologic analysis by electronic microcroscopy of purified CD4+ T cells after 24 hours of incubation with different doses of AG-1478 (µM). No effect of EGFR pharmacological inhibition on nucleus and cytoplasm organisation.

Supplemental figure 3



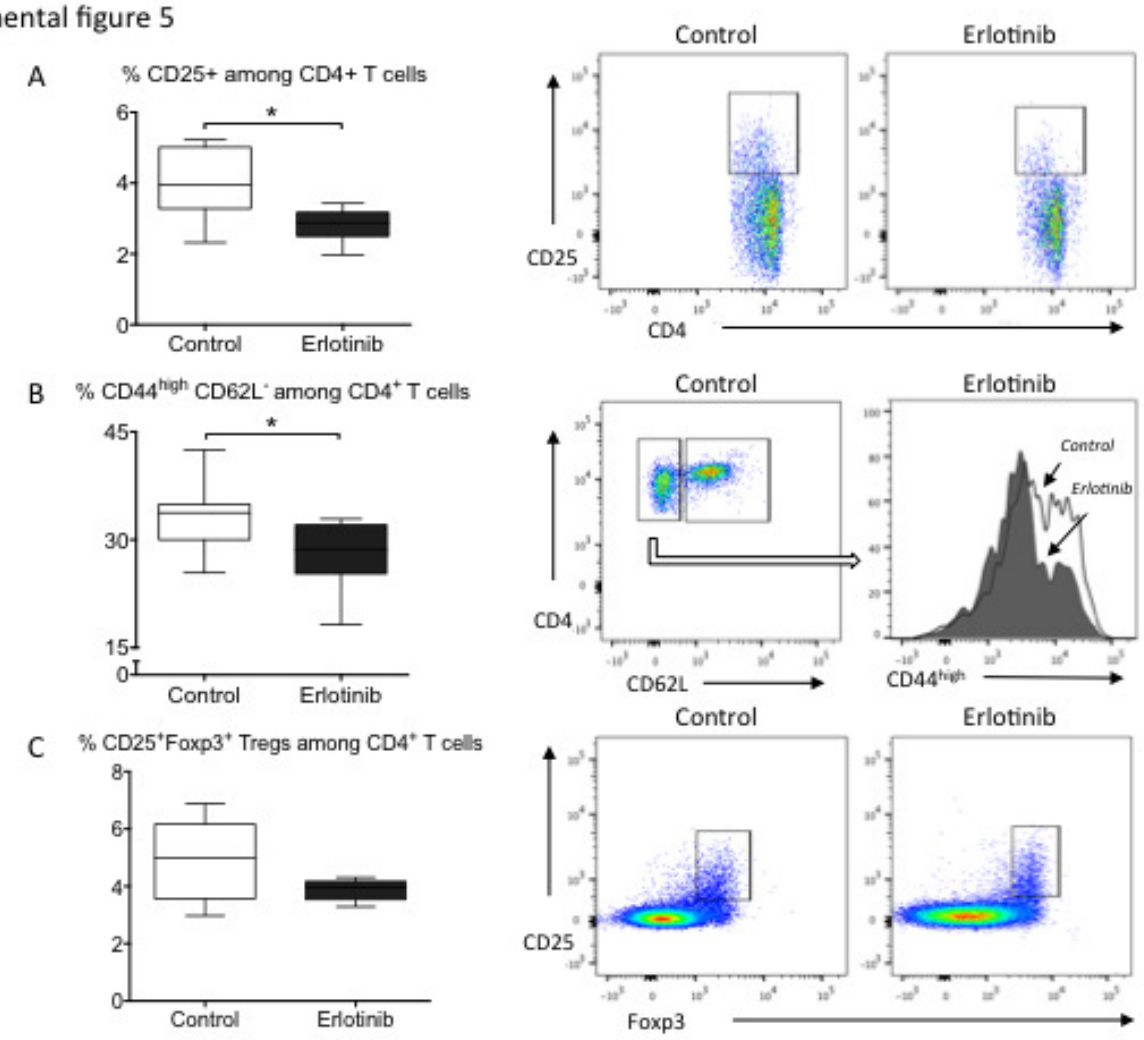
EGFR pharmacological inhibition using AG 1478 reduced IL-2 production by T cells. Splenic CD4+ T cells were stimulated by anti-CD3 coated beads during 48 hours, in the presence or the absence of AG1478 (1µM). A, IL-2 production was measured in the supernatant by ELISA. Data were represented as median and percentiles (5^{th}-95^{th}). (*** $p<0.001$).

Supplemental figure 4



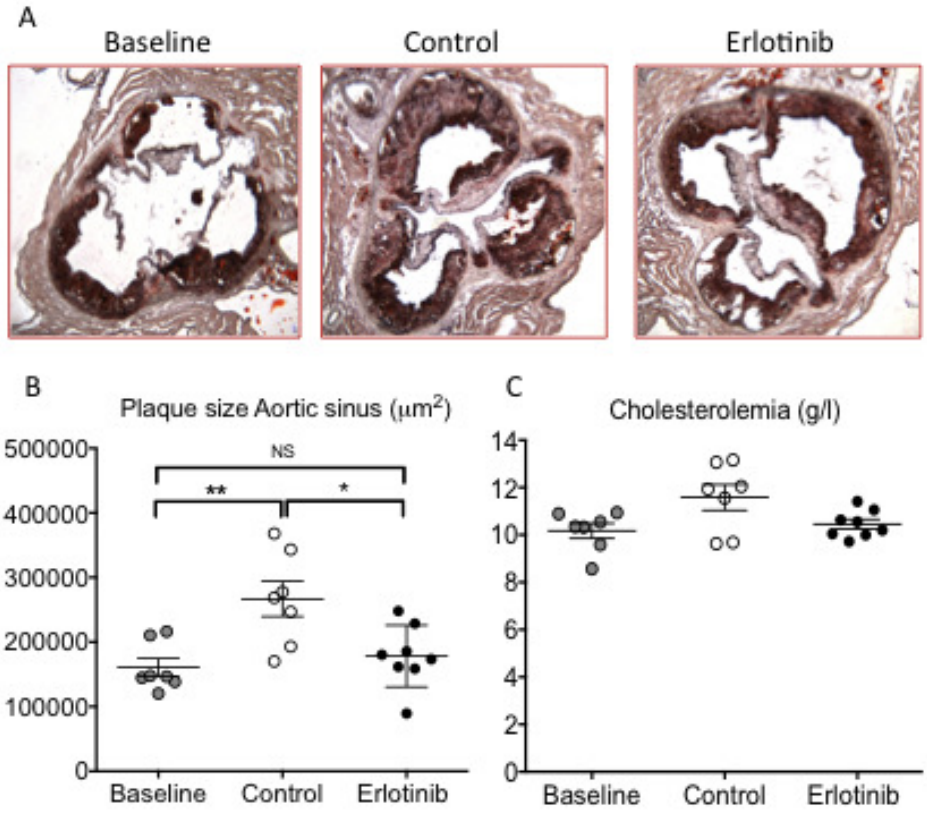
Erlotinib treatment had no effect on animal weight but induced a reduction of spleen size. *Ldlr*$^{-/-}$ mice were daily treated by placebo or Erlotinib (15 mg/kg, orally) during 8 weeks. At sacrifice, mice weight (A) and spleen weight (B). N=8-9/ group. Data were represented as median and percentiles (5^{th}-95^{th}). **, P<0.01

Supplemental figure 5



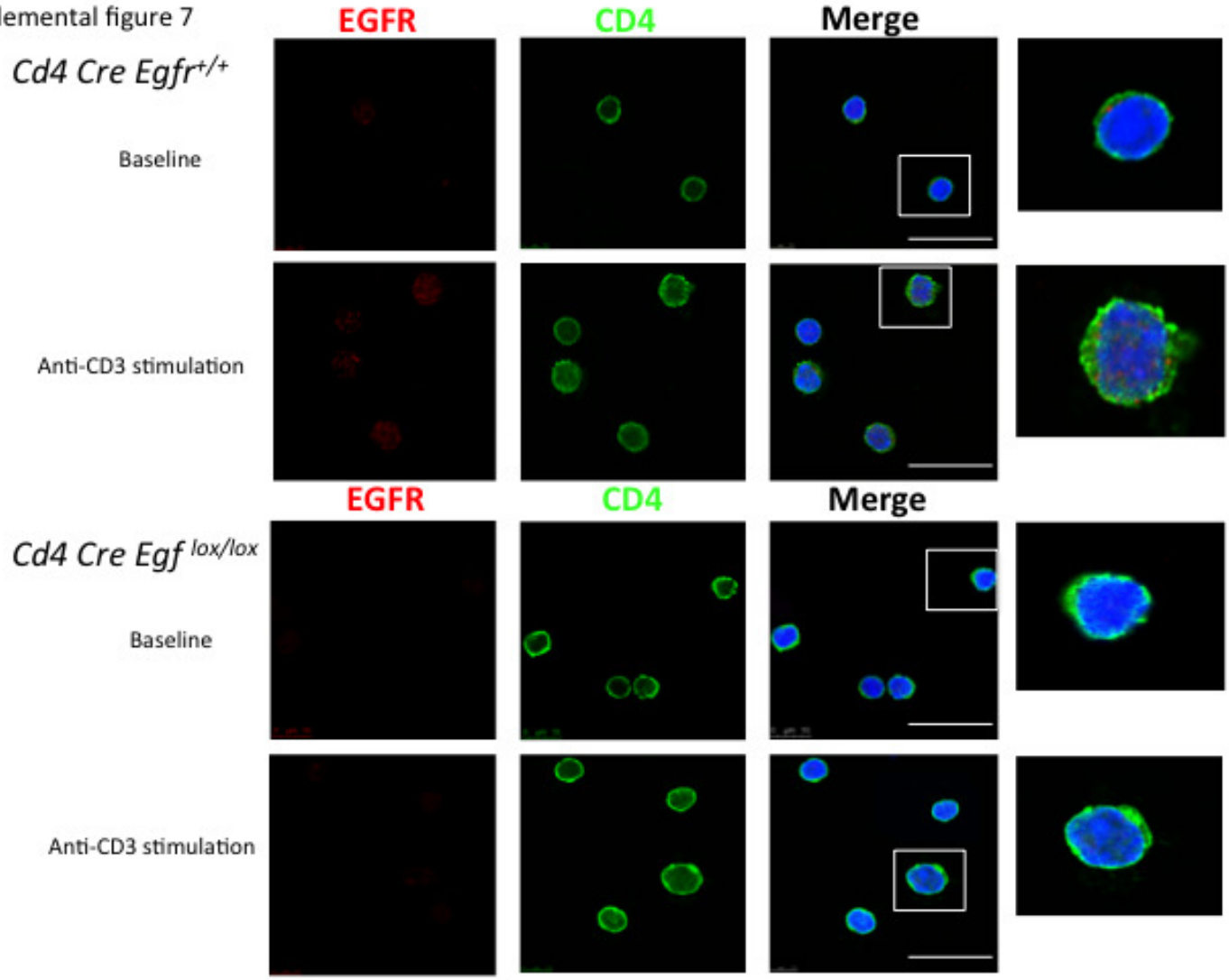
Erlotinib treatment reduced CD4⁺ T cell activation. Representative examples and quantification of CD25 expression by splenic CD4+ T cells (A, gated on CD3+ CD4+ T cells) and $CD44^{high}$ expression by splenic $CD62L^{-}CD4^{+}$ T cells by flow cytometry (B). Representative examples and FACS quantification of $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells among splenic CD4+ T cells (C, gated on CD3+CD4+ T cells). Data were represented as median and percentiles (5^{th}-95^{th}). N=7-8/group, *, P<0.05.

Supplemental figure 6



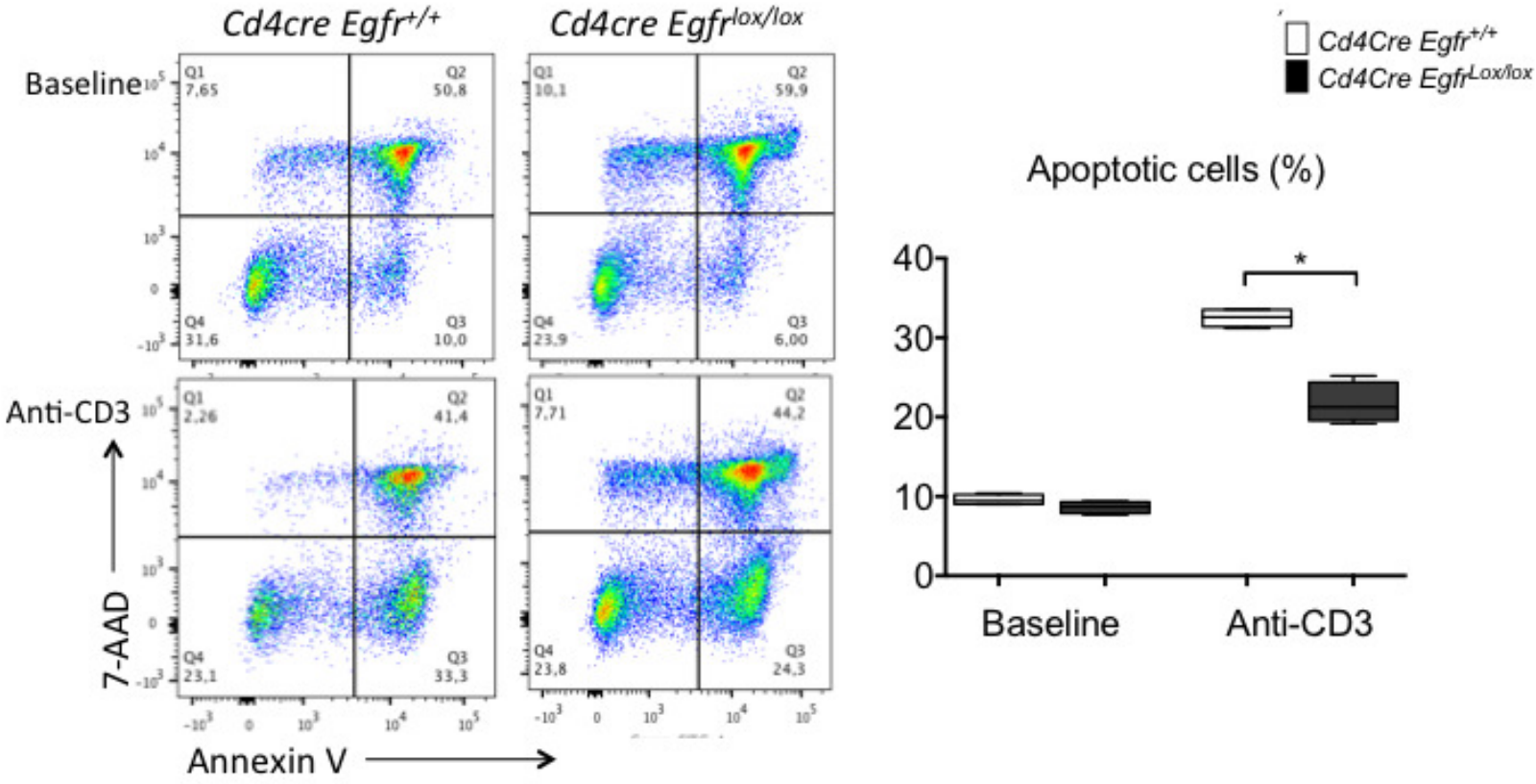
Erlotinib treatment blocked atherosclerosis progression. Representative examples (A) and quantification (B) of atherosclerotic plaque size in the aortic sinus of *Ldlr*$^{-/-}$ mice daily treated by placebo or Erlotinib (15 mg/kg, orally) during 8 weeks following a 8-week period of high fat diet. C, cholesterolemia at sacrifice. N=7-8/group, *, P<0.05, **, P<0.01

Supplemental figure 7



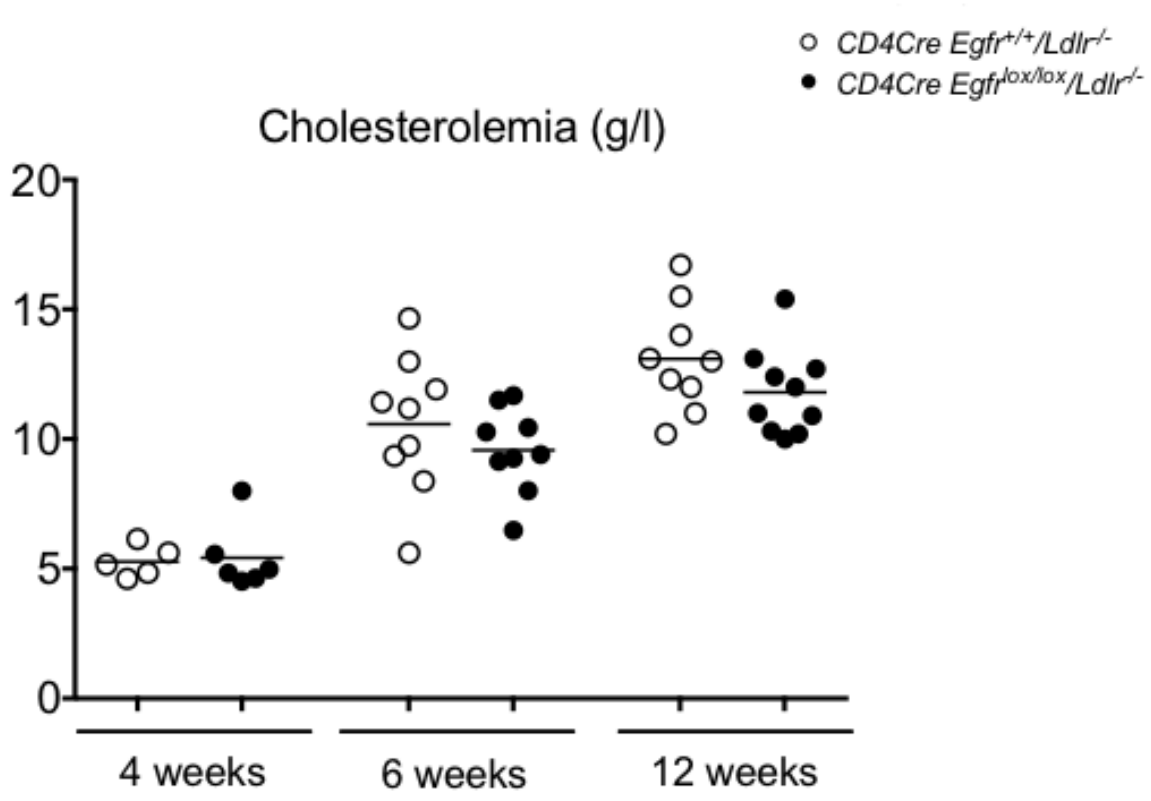
Absence of EGFR expression on CD4+ T cells from *Cd4 Cre Egfr$^{Lox/lox}$* mice. Splenic CD4+ T cells were purified from *Cd4 Cre Egfr$^{+/+}$* and *Cd4 Cre Egfr$^{Lox/lox}$* mice and stimulated with coated anti-CD3 (5 μg/ml) during 24 hours and stained using fluorescent anti-EGFR (Red) and anti-CD4 (Green) antibodies. Bar scale 10 μm.

Supplemental figure 8



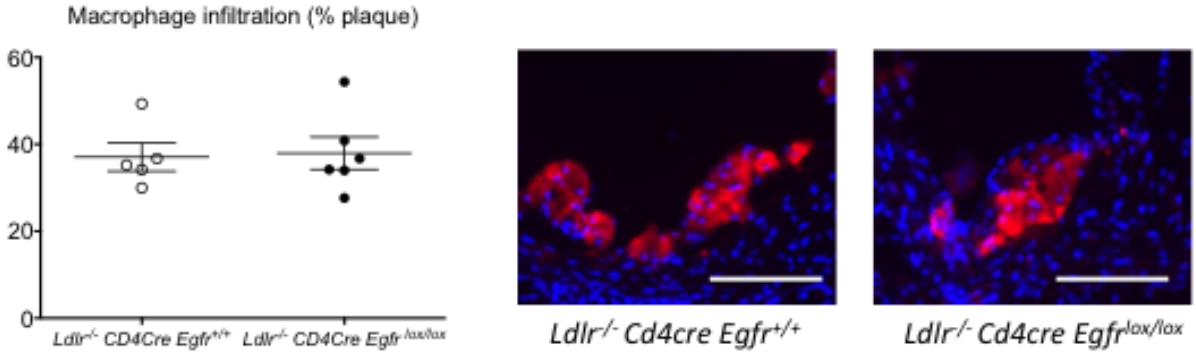
EGFR deficiency reduced T cell apoptosis. Purified CD4+ T cells were isolated from control *Cd4Cre Egfr*$^{+/+}$ and *Cd4Cre Egfr*$^{lox/lox}$ and anti-CD3 stimulated during 48 hours and apoptosis was evaluated by flow cytometry. Apoptotic cells were defined as annexin V^{pos} 7-AAD^{neg} cells. Data were represented as median and percentiles (5th-95th). N=4-5/group, * P<0.05.

Supplemental figure 9



EGFR deficiency had no impact on cholesterolemia. No difference of cholesterolemia between chimeric *Ldlr*$^{-/-}$ mice retransplanted with *Cd4Cre Egfr*$^{+/+}$ *or Cd4Cre Egfr*$^{Lox/lox}$ after 4 (N=5-6/group), 6 (N=9/group) and 12 weeks of fat diet (N=9-10/group).

Supplemental figure 10



EGFR deficiency in T cells had no impact on macrophage content. Quantification and representative photomicrographs of macrophage (MOMA-2+) infiltration in atherosclerotic lesions of irradiated *Ldlr*$^{-/-}$ mice reconstituted with bone marrow from either *Cd4 Cre/Egfr*$^{+/+}$ or *Cd4 Cre/Egfr*$^{lox/lox}$ mice and put on a high fat diet during 6 weeks. Bar scale 50 μm.

 Â

AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
AUTHOR RESPONSIBILITIES: RELATIONSHIP WITH INDUSTRY AND FINANCIAL DISCLOSURE

In addition to disclosing relationship with industry and/or financial disclosures on the *cover letter* and *title* page of your manuscript, we ask that each author sign this form.

To be published in the following journal (please check):

(●) *Journal of the American College of Cardiology(JACC)* ( ) *JACC: Cardiovascular Interventions* ( ) *JACC: Cardiovascular Imaging* ( ) *JACC: Heart Failure* ( ) *JACC: Clinical Electrophysiology* ( ) *JACC: Basic to Translational Science*

Article entitled: Selective EGF-Receptor inhibition in CD4+ T cells induces anergy and limits atherosclerosis

Manuscript number: JACC042617-1616R-A

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: Ait-Oufella Hafid

***Corresponding Author's Responsibility**
To avoid delay in the publication of your manuscript, **complete and submit this form as quickly as possible.**
After you have submitted your electronic form, you will receive an email confirmation that includes a PDF copy. Please print and retain for your files. **If for some reason you are unable to complete this form electronically,** please contact the JACC office directly or print this form, fill in the spaces manually, and email, fax, or post mail a copy to:

Journal of the American College of Cardiology(JACC)
JACC: Cardiovascular Interventions
JACC: Cardiovascular Imaging
JACC: Heart Failure
JACC: Clinical Electrophysiology
JACC: Basic to Translational Science
Heart House, 2400 N Street NW, Washington, DC, 20037
Fax: (202) 375-6819
Email: jacc@acc.org Â jaccint@acc.org Â jaccimg@acc.org Â jacchf@acc.org Â jacccep@acc.org Â jaccbts@acc.org

Complete the section below, type your name into the Type Name box and input the date. *By submitting this form, you attest to being an author on the paper.*

Type Name: Zeboudj LyndaÂ Â Â Â Â Date: 08/09/2017

Title and Company (if employer representative):

I have **no relationship with industry** to disclose.

I have **no financial information** to disclose.

local_p_id: 421984
time: 1504869548
ip address: 193.55.92.45

Â

AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
AUTHOR RESPONSIBILITIES: RELATIONSHIP WITH INDUSTRY AND FINANCIAL DISCLOSURE

In addition to disclosing relationship with industry and/or financial disclosures on the *cover letter* and *title* page of your manuscript, we ask that each author sign this form.

To be published in the following journal (please check):

◉ *Journal of the American College of Cardiology(JACC)* ○ *JACC: Cardiovascular Interventions* ○ *JACC: Cardiovascular Imaging* ○ *JACC: Heart Failure* ○ *JACC: Clinical Electrophysiology* ○ *JACC: Basic to Translational Science*

Article entitled: Selective EGF-Receptor inhibition in CD4+ T cells induces anergy and limits atherosclerosis

Manuscript number: JACC042617-1616RR

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: Dr MAITRE

***Corresponding Author's Responsibility**
To avoid delay in the publication of your manuscript, **complete and submit this form as quickly as possible.**
After you have submitted your electronic form, you will receive an email confirmation that includes a PDF copy. Please print and retain for your files. **If for some reason you are unable to complete this form electronically,** please contact the JACC office directly or print this form, fill in the spaces manually, and email, fax, or post mail a copy to:

Journal of the American College of Cardiology(JACC)
JACC: Cardiovascular Interventions
JACC: Cardiovascular Imaging
JACC: Heart Failure
JACC: Clinical Electrophysiology
JACC: Basic to Translational Science
Heart House, 2400 N Street NW, Washington, DC, 20037
Fax: (202) 375-6819
Email: jacc@acc.org Â jaccint@acc.org Â jaccimg@acc.org Â jacchf@acc.org Â jacccep@acc.org Â jaccbts@acc.org

Complete the section below, type your name into the Type Name box and input the date. *By submitting this form, you attest to being an author on the paper.*

Type Name: MAITRE MIKAELÂ Â Â Â Â Date: 10/15/2017

Title and Company (if employer representative): PhD

I have **no relationship with industry** to disclose.

I have **no financial information** to disclose.

local_p_id: 448143
time: 1508064002
ip address: 37.172.95.116

 Â

AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
AUTHOR RESPONSIBILITIES: RELATIONSHIP WITH INDUSTRY AND FINANCIAL DISCLOSURE

In addition to disclosing relationship with industry and/or financial disclosures on the *cover letter* and *title* page of your manuscript, we ask that each author sign this form.

To be published in the following journal (please check):

(●) *Journal of the American College of Cardiology(JACC)* ( ) *JACC: Cardiovascular Interventions* ( ) *JACC: Cardiovascular Imaging* ( ) *JACC: Heart Failure* ( ) *JACC: Clinical Electrophysiology* ( ) *JACC: Basic to Translational Science*

Article entitled: Selective EGF-Receptor inhibition in CD4+ T cells induces anergy and limits atherosclerosis

Manuscript number: JACC042617-1616RR

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: Ait Oufella

***Corresponding Author's Responsibility**
To avoid delay in the publication of your manuscript, **complete and submit this form as quickly as possible.**
After you have submitted your electronic form, you will receive an email confirmation that includes a PDF copy. Please print and retain for your files. **If for some reason you are unable to complete this form electronically,** please contact the JACC office directly or print this form, fill in the spaces manually, and email, fax, or post mail a copy to:

Journal of the American College of Cardiology(JACC)
JACC: Cardiovascular Interventions
JACC: Cardiovascular Imaging
JACC: Heart Failure
JACC: Clinical Electrophysiology
JACC: Basic to Translational Science
Heart House, 2400 N Street NW, Washington, DC, 20037
Fax: (202) 375-6819
Email: jacc@acc.org Â jaccint@acc.org Â jaccimg@acc.org Â jacchf@acc.org Â jacccep@acc.org Â jaccbts@acc.org

Complete the section below, type your name into the Type Name box and input the date. *By submitting this form, you attest to being an author on the paper.*

Type Name: GuyonnetÂ Â Â Â Â Â Date: 14/09/17

Title and Company (if employer representative): PhD

I have **no relationship with industry** to disclose.

I have **no financial information** to disclose.

local_p_id: 448144
time: 1505382263
ip address: 85.68.142.161

 Â

AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
AUTHOR RESPONSIBILITIES: RELATIONSHIP WITH INDUSTRY AND FINANCIAL DISCLOSURE

In addition to disclosing relationship with industry and/or financial disclosures on the *cover letter* and *title* page of your manuscript, we ask that each author sign this form.

To be published in the following journal (please check):

(●) *Journal of the American College of Cardiology(JACC)* ( ) *JACC: Cardiovascular Interventions* ( ) *JACC: Cardiovascular Imaging* ( ) *JACC: Heart Failure* ( ) *JACC: Clinical Electrophysiology* ( ) *JACC: Basic to Translational Science*

Article entitled: Selective EGF-Receptor inhibition in CD4+ T cells induces anergy and limits atherosclerosis

Manuscript number: JACC042617-1616RR

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: Ait-Oufella

***Corresponding Author's Responsibility**
To avoid delay in the publication of your manuscript, **complete and submit this form as quickly as possible.**
After you have submitted your electronic form, you will receive an email confirmation that includes a PDF copy. Please print and retain for your files. **If for some reason you are unable to complete this form electronically,** please contact the JACC office directly or print this form, fill in the spaces manually, and email, fax, or post mail a copy to:

Journal of the American College of Cardiology(JACC)
JACC: Cardiovascular Interventions
JACC: Cardiovascular Imaging
JACC: Heart Failure
JACC: Clinical Electrophysiology
JACC: Basic to Translational Science
Heart House, 2400 N Street NW, Washington, DC, 20037
Fax: (202) 375-6819
Email: jacc@acc.org Â jaccint@acc.org Â jaccimg@acc.org Â jacchf@acc.org Â jacccep@acc.org Â jaccbts@acc.org

Complete the section below, type your name into the Type Name box and input the date. *By submitting this form, you attest to being an author on the paper.*

Type Name: LaransÂ Â Â Â Â Date: 09/12/17

Title and Company (if employer representative): Inserm

I have **no relationship with industry** to disclose.

I have **no financial information** to disclose.

local_p_id: 291715
time: 1505225055
ip address: 193.55.92.45

 Â

AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
AUTHOR RESPONSIBILITIES: RELATIONSHIP WITH INDUSTRY AND FINANCIAL DISCLOSURE

In addition to disclosing relationship with industry and/or financial disclosures on the *cover letter* and *title* page of your manuscript, we ask that each author sign this form.

To be published in the following journal (please check):

(●) *Journal of the American College of Cardiology(JACC)* ( ) *JACC: Cardiovascular Interventions* ( ) *JACC: Cardiovascular Imaging* ( ) *JACC: Heart Failure* ( ) *JACC: Clinical Electrophysiology* ( ) *JACC: Basic to Translational Science*

Article entitled: Selective EGF-Receptor inhibition in CD4+ T cells induces anergy and limits atherosclerosis

Manuscript number: JACC042617-1616R-A

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: Joffre

***Corresponding Author's Responsibility**
To avoid delay in the publication of your manuscript, **complete and submit this form as quickly as possible.**
After you have submitted your electronic form, you will receive an email confirmation that includes a PDF copy. Please print and retain for your files. **If for some reason you are unable to complete this form electronically,** please contact the JACC office directly or print this form, fill in the spaces manually, and email, fax, or post mail a copy to:

Journal of the American College of Cardiology(JACC)
JACC: Cardiovascular Interventions
JACC: Cardiovascular Imaging
JACC: Heart Failure
JACC: Clinical Electrophysiology
JACC: Basic to Translational Science
Heart House, 2400 N Street NW, Washington, DC, 20037
Fax: (202) 375-6819
Email: jacc@acc.org Â jaccint@acc.org Â jaccimg@acc.org Â jacchf@acc.org Â jacccep@acc.org Â jaccbts@acc.org

Complete the section below, type your name into the Type Name box and input the date. *By submitting this form, you attest to being an author on the paper.*

Type Name: JoffreÂ Â Â Â Â Date: 09/08/2017

Title and Company (if employer representative): MD. PhD

I have **no relationship with industry** to disclose.

I have **no financial information** to disclose.

local_p_id: 354880
time: 1504865740
ip address: 37.170.253.1

Â

AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
AUTHOR RESPONSIBILITIES: RELATIONSHIP WITH INDUSTRY AND FINANCIAL DISCLOSURE

In addition to disclosing relationship with industry and/or financial disclosures on the *cover letter* and *title* page of your manuscript, we ask that each author sign this form.

To be published in the following journal (please check):

◉ *Journal of the American College of Cardiology(JACC)* ○ *JACC: Cardiovascular Interventions* ○ *JACC: Cardiovascular Imaging* ○ *JACC: Heart Failure* ○ *JACC: Clinical Electrophysiology* ○ *JACC: Basic to Translational Science*

Article entitled: Selective EGF-Receptor inhibition in CD4+ T cells induces anergy and limits atherosclerosis

Manuscript number: JACC042617-1616RR

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: Prof. Ait oufella

***Corresponding Author's Responsibility**
To avoid delay in the publication of your manuscript, **complete and submit this form as quickly as possible.**
After you have submitted your electronic form, you will receive an email confirmation that includes a PDF copy. Please print and retain for your files. **If for some reason you are unable to complete this form electronically,** please contact the JACC office directly or print this form, fill in the spaces manually, and email, fax, or post mail a copy to:

Journal of the American College of Cardiology(JACC)
JACC: Cardiovascular Interventions
JACC: Cardiovascular Imaging
JACC: Heart Failure
JACC: Clinical Electrophysiology
JACC: Basic to Translational Science
Heart House, 2400 N Street NW, Washington, DC, 20037
Fax: (202) 375-6819
Email: jacc@acc.org Â jaccint@acc.org Â jaccimg@acc.org Â jacchf@acc.org Â jacccep@acc.org Â jaccbts@acc.org

Complete the section below, type your name into the Type Name box and input the date. *By submitting this form, you attest to being an author on the paper.*

Type Name: Lemarie JérémieÂ Â Â Â Â Date: 15/10/2017

Title and Company (if employer representative):

I have **no relationship with industry** to disclose.

I have **no financial information** to disclose.

local_p_id: 448145
time: 1508052796
ip address: 37.58.146.117

 Â

AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
AUTHOR RESPONSIBILITIES: RELATIONSHIP WITH INDUSTRY AND FINANCIAL DISCLOSURE

In addition to disclosing relationship with industry and/or financial disclosures on the *cover letter* and *title* page of your manuscript, we ask that each author sign this form.

To be published in the following journal (please check):

(●) *Journal of the American College of Cardiology(JACC)* ( ) *JACC: Cardiovascular Interventions* ( ) *JACC: Cardiovascular Imaging* ( ) *JACC: Heart Failure* ( ) *JACC: Clinical Electrophysiology* ( ) *JACC: Basic to Translational Science*

Article entitled: Selective EGF-Receptor inhibition in CD4+ T cells induces anergy and limits atherosclerosis

Manuscript number: JACC042617-1616RR

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: Simon Bourcier

***Corresponding Author's Responsibility**
To avoid delay in the publication of your manuscript, **complete and submit this form as quickly as possible.**
After you have submitted your electronic form, you will receive an email confirmation that includes a PDF copy. Please print and retain for your files. **If for some reason you are unable to complete this form electronically,** please contact the JACC office directly or print this form, fill in the spaces manually, and email, fax, or post mail a copy to:

Journal of the American College of Cardiology(JACC)
JACC: Cardiovascular Interventions
JACC: Cardiovascular Imaging
JACC: Heart Failure
JACC: Clinical Electrophysiology
JACC: Basic to Translational Science
Heart House, 2400 N Street NW, Washington, DC, 20037
Fax: (202) 375-6819
Email: jacc@acc.org Â jaccint@acc.org Â jaccimg@acc.org Â jacchf@acc.org Â jacccep@acc.org Â jaccbts@acc.org

Complete the section below, type your name into the Type Name box and input the date. *By submitting this form, you attest to being an author on the paper.*

Type Name: BourcierÂ Â Â Â Â Date: 10/15/2017

Title and Company (if employer representative):

I have **no relationship with industry** to disclose.

I have **no financial information** to disclose.

local_p_id: 438779
time: 1508053456
ip address: 84.102.146.233

Â

AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
AUTHOR RESPONSIBILITIES: RELATIONSHIP WITH INDUSTRY AND FINANCIAL DISCLOSURE

In addition to disclosing relationship with industry and/or financial disclosures on the *cover letter* and *title* page of your manuscript, we ask that each author sign this form.

To be published in the following journal (please check):

◉ *Journal of the American College of Cardiology(JACC)* ○ *JACC: Cardiovascular Interventions* ○ *JACC: Cardiovascular Imaging* ○ *JACC: Heart Failure* ○ *JACC: Clinical Electrophysiology* ○ *JACC: Basic to Translational Science*

Article entitled: Selective EGF-Receptor inhibition in CD4+ T cells induces anergy and limits atherosclerosis

Manuscript number: JACC042617-1616RR

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: Hafid Ait Oufella

***Corresponding Author's Responsibility**
To avoid delay in the publication of your manuscript, **complete and submit this form as quickly as possible.**
After you have submitted your electronic form, you will receive an email confirmation that includes a PDF copy. Please print and retain for your files. **If for some reason you are unable to complete this form electronically,** please contact the JACC office directly or print this form, fill in the spaces manually, and email, fax, or post mail a copy to:

Journal of the American College of Cardiology(JACC)
JACC: Cardiovascular Interventions
JACC: Cardiovascular Imaging
JACC: Heart Failure
JACC: Clinical Electrophysiology
JACC: Basic to Translational Science
Heart House, 2400 N Street NW, Washington, DC, 20037
Fax: (202) 375-6819
Email: jacc@acc.org Â jaccint@acc.org Â jaccimg@acc.org Â jacchf@acc.org Â jacccep@acc.org Â jaccbts@acc.org

Complete the section below, type your name into the Type Name box and input the date. *By submitting this form, you attest to being an author on the paper.*

Type Name: Wared Nour-EldineÂ Â Â Â Â Date: 01 11 2017

Title and Company (if employer representative):

I have **no relationship with industry** to disclose.

I have **no financial information** to disclose.

local_p_id: 448146
time: 1509542620
ip address: 194.146.155.130

Â

AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
AUTHOR RESPONSIBILITIES: RELATIONSHIP WITH INDUSTRY AND FINANCIAL DISCLOSURE

In addition to disclosing relationship with industry and/or financial disclosures on the *cover letter* and *title* page of your manuscript, we ask that each author sign this form.

To be published in the following journal (please check):

◉ *Journal of the American College of Cardiology(JACC)* ○ *JACC: Cardiovascular Interventions* ○ *JACC: Cardiovascular Imaging* ○ *JACC: Heart Failure* ○ *JACC: Clinical Electrophysiology* ○ *JACC: Basic to Translational Science*

Article entitled: Selective EGF-Receptor inhibition in CD4+ T cells induces anergy and limits atherosclerosis

Manuscript number: JACC042617-1616R-A

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: Ait Oufella

***Corresponding Author's Responsibility**
To avoid delay in the publication of your manuscript, **complete and submit this form as quickly as possible.**
After you have submitted your electronic form, you will receive an email confirmation that includes a PDF copy. Please print and retain for your files. **If for some reason you are unable to complete this form electronically,** please contact the JACC office directly or print this form, fill in the spaces manually, and email, fax, or post mail a copy to:

Journal of the American College of Cardiology(JACC)
JACC: Cardiovascular Interventions
JACC: Cardiovascular Imaging
JACC: Heart Failure
JACC: Clinical Electrophysiology
JACC: Basic to Translational Science
Heart House, 2400 N Street NW, Washington, DC, 20037
Fax: (202) 375-6819
Email: jacc@acc.org Â jaccint@acc.org Â jaccimg@acc.org Â jacchf@acc.org Â jacccep@acc.org Â jaccbts@acc.org

Complete the section below, type your name into the Type Name box and input the date. *By submitting this form, you attest to being an author on the paper.*

Type Name: Jonas FRIARDÂ Â Â Â Â Date: 08 September 2017

Title and Company (if employer representative): LP2M CNRS-UMR7370, Medical Faculty, UniversitÃ© CÃ´te dâ Azur, Nice, France

☐ I have **no relationship with industry** to disclose.

☐ I have **no financial information** to disclose.

local_p_id: 448147
time: 1504865124
ip address: 134.59.54.186

Â

AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
AUTHOR RESPONSIBILITIES: RELATIONSHIP WITH INDUSTRY AND FINANCIAL DISCLOSURE

In addition to disclosing relationship with industry and/or financial disclosures on the *cover letter* and *title* page of your manuscript, we ask that each author sign this form.

To be published in the following journal (please check):

◉ *Journal of the American College of Cardiology(JACC)* ○ *JACC: Cardiovascular Interventions* ○ *JACC: Cardiovascular Imaging* ○ *JACC: Heart Failure* ○ *JACC: Clinical Electrophysiology* ○ *JACC: Basic to Translational Science*

Article entitled: Selective EGF-Receptor inhibition in CD4+ T cells induces anergy and limits atherosclerosis

Manuscript number: JACC042617-1616R-A

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: Ait Oufella

***Corresponding Author's Responsibility**
To avoid delay in the publication of your manuscript, **complete and submit this form as quickly as possible.**
After you have submitted your electronic form, you will receive an email confirmation that includes a PDF copy. Please print and retain for your files. **If for some reason you are unable to complete this form electronically,** please contact the JACC office directly or print this form, fill in the spaces manually, and email, fax, or post mail a copy to:

Journal of the American College of Cardiology(JACC)
JACC: Cardiovascular Interventions
JACC: Cardiovascular Imaging
JACC: Heart Failure
JACC: Clinical Electrophysiology
JACC: Basic to Translational Science
Heart House, 2400 N Street NW, Washington, DC, 20037
Fax: (202) 375-6819
Email: jacc@acc.org Â jaccint@acc.org Â jaccimg@acc.org Â jacchf@acc.org Â jacccep@acc.org Â jaccbts@acc.org

Complete the section below, type your name into the Type Name box and input the date. *By submitting this form, you attest to being an author on the paper.*

Type Name: WakkachÂ Â Â Â Â Date: 09/08/2017

Title and Company (if employer representative): PhD

I have **no relationship with industry** to disclose.

I have **no financial information** to disclose.

local_p_id: 448148
time: 1504869219
ip address: 134.59.54.154

Â

AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
AUTHOR RESPONSIBILITIES: RELATIONSHIP WITH INDUSTRY AND FINANCIAL DISCLOSURE

In addition to disclosing relationship with industry and/or financial disclosures on the *cover letter* and *title* page of your manuscript, we ask that each author sign this form.

To be published in the following journal (please check):

(●) *Journal of the American College of Cardiology(JACC)* ( ) *JACC: Cardiovascular Interventions* ( ) *JACC: Cardiovascular Imaging* ( ) *JACC: Heart Failure* ( ) *JACC: Clinical Electrophysiology* ( ) *JACC: Basic to Translational Science*

Article entitled: Selective EGF-Receptor inhibition in CD4+ T cells induces anergy and limits atherosclerosis

Manuscript number: JACC042617-1616RR

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: Hafid Aitoufella

***Corresponding Author's Responsibility**
To avoid delay in the publication of your manuscript, **complete and submit this form as quickly as possible.**
After you have submitted your electronic form, you will receive an email confirmation that includes a PDF copy. Please print and retain for your files. **If for some reason you are unable to complete this form electronically,** please contact the JACC office directly or print this form, fill in the spaces manually, and email, fax, or post mail a copy to:

Journal of the American College of Cardiology(JACC)
JACC: Cardiovascular Interventions
JACC: Cardiovascular Imaging
JACC: Heart Failure
JACC: Clinical Electrophysiology
JACC: Basic to Translational Science
Heart House, 2400 N Street NW, Washington, DC, 20037
Fax: (202) 375-6819
Email: jacc@acc.org Â jaccint@acc.org Â jaccimg@acc.org Â jacchf@acc.org Â jacccep@acc.org Â jaccbts@acc.org

Complete the section below, type your name into the Type Name box and input the date. *By submitting this form, you attest to being an author on the paper.*

Type Name: FABRE ElizabethÂ Â Â Â Â Date: 15/10/2017

Title and Company (if employer representative): MD,PhD. Hôpital européen Georges Pompidou

I have **no relationship with industry** to disclose.

I have **no financial information** to disclose.

local_p_id: 457223
time: 1508145101
ip address: 164.2.255.244

Â

AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
AUTHOR RESPONSIBILITIES: RELATIONSHIP WITH INDUSTRY AND FINANCIAL DISCLOSURE

In addition to disclosing relationship with industry and/or financial disclosures on the *cover letter* and *title* page of your manuscript, we ask that each author sign this form.

To be published in the following journal (please check):

◉ *Journal of the American College of Cardiology(JACC)* ○ *JACC: Cardiovascular Interventions* ○ *JACC: Cardiovascular Imaging* ○ *JACC: Heart Failure* ○ *JACC: Clinical Electrophysiology* ○ *JACC: Basic to Translational Science*

Article entitled: Selective EGF-Receptor inhibition in CD4+ T cells induces anergy and limits atherosclerosis

Manuscript number: JACC042617-1616R-A

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: Hafid AIT-OUFELLA

***Corresponding Author's Responsibility**
To avoid delay in the publication of your manuscript, **complete and submit this form as quickly as possible.**
After you have submitted your electronic form, you will receive an email confirmation that includes a PDF copy. Please print and retain for your files. **If for some reason you are unable to complete this form electronically,** please contact the JACC office directly or print this form, fill in the spaces manually, and email, fax, or post mail a copy to:

Journal of the American College of Cardiology(JACC)
JACC: Cardiovascular Interventions
JACC: Cardiovascular Imaging
JACC: Heart Failure
JACC: Clinical Electrophysiology
JACC: Basic to Translational Science
Heart House, 2400 N Street NW, Washington, DC, 20037
Fax: (202) 375-6819
Email: jacc@acc.org Â jaccint@acc.org Â jaccimg@acc.org Â jacchf@acc.org Â jacccep@acc.org Â jaccbts@acc.org

Complete the section below, type your name into the Type Name box and input the date. *By submitting this form, you attest to being an author on the paper.*

Type Name: Alain TEDGUIÂ Â Â Â Â Date: 09/08/17

Title and Company (if employer representative):

I have **no relationship with industry** to disclose.

I have **no financial information** to disclose.

local_p_id: 448149
time: 1504867488
ip address: 193.55.92.35

Â

AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
AUTHOR RESPONSIBILITIES: RELATIONSHIP WITH INDUSTRY AND FINANCIAL DISCLOSURE

In addition to disclosing relationship with industry and/or financial disclosures on the *cover letter* and *title* page of your manuscript, we ask that each author sign this form.

To be published in the following journal (please check):

(●) *Journal of the American College of Cardiology(JACC)* ( ) *JACC: Cardiovascular Interventions* ( ) *JACC: Cardiovascular Imaging* ( ) *JACC: Heart Failure* ( ) *JACC: Clinical Electrophysiology* ( ) *JACC: Basic to Translational Science*

Article entitled: Selective EGF-Receptor inhibition in CD4+ T cells induces anergy and limits atherosclerosis

Manuscript number: JACC042617-1616R-A

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: Ait-Oufella

***Corresponding Author's Responsibility**
To avoid delay in the publication of your manuscript, **complete and submit this form as quickly as possible.**
After you have submitted your electronic form, you will receive an email confirmation that includes a PDF copy. Please print and retain for your files. **If for some reason you are unable to complete this form electronically,** please contact the JACC office directly or print this form, fill in the spaces manually, and email, fax, or post mail a copy to:

Journal of the American College of Cardiology(JACC)
JACC: Cardiovascular Interventions
JACC: Cardiovascular Imaging
JACC: Heart Failure
JACC: Clinical Electrophysiology
JACC: Basic to Translational Science
Heart House, 2400 N Street NW, Washington, DC, 20037
Fax: (202) 375-6819
Email: jacc@acc.org Â jaccint@acc.org Â jaccimg@acc.org Â jacchf@acc.org Â jacccep@acc.org Â jaccbts@acc.org

Complete the section below, type your name into the Type Name box and input the date. *By submitting this form, you attest to being an author on the paper.*

Type Name: Ziad MallatÂ Â Â Â Â Date: 08/09/2017

Title and Company (if employer representative): University of Cambridge

I have **no relationship with industry** to disclose.

I have **no financial information** to disclose.

local_p_id: 421999
time: 1504864359
ip address: 193.55.92.45

 Â

AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
AUTHOR RESPONSIBILITIES: RELATIONSHIP WITH INDUSTRY AND FINANCIAL DISCLOSURE

In addition to disclosing relationship with industry and/or financial disclosures on the *cover letter* and *title* page of your manuscript, we ask that each author sign this form.

To be published in the following journal (please check):

(●) *Journal of the American College of Cardiology(JACC)* ( ) *JACC: Cardiovascular Interventions* ( ) *JACC: Cardiovascular Imaging* ( ) *JACC: Heart Failure* ( ) *JACC: Clinical Electrophysiology* ( ) *JACC: Basic to Translational Science*

Article entitled: Selective EGF-Receptor inhibition in CD4+ T cells induces anergy and limits atherosclerosis

Manuscript number: JACC042617-1616R-A

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: AIT OUFELLA and THARAUX

***Corresponding Author's Responsibility**
To avoid delay in the publication of your manuscript, **complete and submit this form as quickly as possible.**
After you have submitted your electronic form, you will receive an email confirmation that includes a PDF copy. Please print and retain for your files. **If for some reason you are unable to complete this form electronically,** please contact the JACC office directly or print this form, fill in the spaces manually, and email, fax, or post mail a copy to:

Journal of the American College of Cardiology(JACC)
JACC: Cardiovascular Interventions
JACC: Cardiovascular Imaging
JACC: Heart Failure
JACC: Clinical Electrophysiology
JACC: Basic to Translational Science
Heart House, 2400 N Street NW, Washington, DC, 20037
Fax: (202) 375-6819
Email: jacc@acc.org Â jaccint@acc.org Â jaccimg@acc.org Â jacchf@acc.org Â jacccep@acc.org Â jaccbts@acc.org

Complete the section below, type your name into the Type Name box and input the date. *By submitting this form, you attest to being an author on the paper.*

Type Name: TharauxÂ Â Â Â Â Date: 09/08/2107

Title and Company (if employer representative): Inserm

I have **no relationship with industry** to disclose.

I have **no financial information** to disclose.

local_p_id: 448150
time: 1504866018
ip address: 193.55.92.48

 Â

AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
AUTHOR RESPONSIBILITIES: RELATIONSHIP WITH INDUSTRY AND FINANCIAL DISCLOSURE

In addition to disclosing relationship with industry and/or financial disclosures on the *cover letter* and *title* page of your manuscript, we ask that each author sign this form.

To be published in the following journal (please check):

(●) *Journal of the American College of Cardiology(JACC)* ( ) *JACC: Cardiovascular Interventions* ( ) *JACC: Cardiovascular Imaging* ( ) *JACC: Heart Failure* ( ) *JACC: Clinical Electrophysiology* ( ) *JACC: Basic to Translational Science*

Article entitled: Selective EGF-Receptor inhibition in CD4+ T cells induces anergy and limits atherosclerosis

Manuscript number: JACC042617-1616RR

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: Ait-Oufella

***Corresponding Author's Responsibility**
To avoid delay in the publication of your manuscript, **complete and submit this form as quickly as possible.**
After you have submitted your electronic form, you will receive an email confirmation that includes a PDF copy. Please print and retain for your files. **If for some reason you are unable to complete this form electronically,** please contact the JACC office directly or print this form, fill in the spaces manually, and email, fax, or post mail a copy to:

Journal of the American College of Cardiology(JACC)
JACC: Cardiovascular Interventions
JACC: Cardiovascular Imaging
JACC: Heart Failure
JACC: Clinical Electrophysiology
JACC: Basic to Translational Science
Heart House, 2400 N Street NW, Washington, DC, 20037
Fax: (202) 375-6819
Email: jacc@acc.org Â jaccint@acc.org Â jaccimg@acc.org Â jacchf@acc.org Â jacccep@acc.org Â jaccbts@acc.org

Complete the section below, type your name into the Type Name box and input the date. *By submitting this form, you attest to being an author on the paper.*

Type Name: Ait-OufellaÂ Â Â Â Â Date: 09/09/17

Title and Company (if employer representative): Professor, Inserm

I have **no relationship with industry** to disclose.

I have **no financial information** to disclose.

local_p_id: 421977
time: 1504949058
ip address: 92.90.20.133